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BIOLOGICAL BULLETIN

OF THE

Marine Biological Laboratory

WOODS HOLE, MASS.

VOL. LVII AUGUST, 1929 NO. 2

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PUBLISHED MONTHLY BY THE
MARINE BIOLOGICAL LABORATORY

PRINTED AND ISSUED BY
LANCASTER PRESS, INC.
LANCASTER, PA.

AGENT FOR GREAT BRITAIN
WHELDON & WESLEY, LIMITED
2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2

Single Numbers, $1.00. Per Volume (6 numbers), $4.50

Entered October 10, 1902, at Lancaster, Pa., as second-class matter under Act of Congress of July 16, 1894

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Vol. LVII. *August, 1929* *No. 2*

BIOLOGICAL BULLETIN

UROLEPTUS HALSEYI, N. SP. I. THE EFFECT OF ULTRA-VIOLET RAYS.

GARY N. CALKINS,

COLUMBIA UNIVERSITY, NEW YORK CITY.

Twelve years ago I found an organism which I described as a variety of *Uroleptus mobilis* Engelmann.[1] The origin of the culture which had been standing in the laboratory for four months was unknown. The organism was cultivated and studied for a period of ten years by the isolation culture method. It is evidently much more rare than *Uroleptus rattulus* or *U. pisces* for I have been unable to find any reference to it since its discovery by Engelmann in 1862.

Late in the fall of 1928 Mr. H. R. Halsey called my attention to a ciliate which he believed to be *Uroleptus mobilis* which he had found in one of the general class cultures. He had collected the material from a pond in Westchester County where sphagnum, *Myriophyllum*, etc., were abundant. The organism was present in great abundance and study soon showed that it was not *U. mobilis* but a closely related new species which I am very glad to name after my friend and assistant—*Uroleptus Halseyi.*

The two species are quite similar in general character and appearance and may be mistaken easily one for the other (Figs. 1, 2).

Engelmann's original description of *Uroleptus mobilis* runs as follows:

Body form constant; plastic; circular in section; about twelve times longer than broad; tapering gradually to a broadly pointed posterior end. Lateral cilia equally long throughout. With six elongate nuclei. This species which came from the Boticzbach

[1] *Uroleptus mobilis* Engelmann. I. History of the Nuclei during Division and Conjugation. Jour. Expt. Zoöl., Vol. 27, 1919.

near Prag, is distinguished from the other species of *Uroleptus* recently described by Stein, by the constant presence of six nuclei arranged one behind the other. It stands close to Stein's *Uroleptus rattulus* but, unlike this species, it possesses no sharply pointed posterior end, and the lateral cilia are equally long on the tail and body. The adoral row of cilia (adoral zone) occupies about one ninth of the total body length, with an undulating membrane fastened on its inner side; a peristome field appears to be entirely absent, or at least extremely narrow. Whether, as in other *Uroleptus* species, two longitudinal rows of fine ventral cilia are present, I could not make out since the animal is very lively and inclined to creep about, snake-like, between plant remains. Our species moreover, which appeared in great numbers, measured on the average 0.30 mm. All specimens were about of the same size (*loc. cit.*, p. 386).

The New York type of this species does not agree in all details with this original description as shown by the following account:

"Body form constant, plastic, circular in section; about 10.5 times longer than broad and tapering gradually to a broadly pointed posterior end. The posterior end is permanently curved towards the ventral side. (Engelmann does not mention this curvature but represents it in his figure.) The lateral cilia are long and distinct and sparsely distributed in straight rows running from end to end and around the posterior end of the body. Owing to density of the protoplasm the ventral cilia cannot be made out on the living organism but in cross sections it is evident that three rows of ventral cilia are present, and that they are finer and shorter than the lateral cilia. The frontal cirri, three in number, are placed in an oblique row at the extreme anterior end of the ventral surface. The terminal cilia of the ventral rows are conspicuous at the anterior end and give the impression of five or six frontal cirri. The peristome occupies about one sixth of the entire length of the body and this region is slightly flattened. The peristome is very narrow with an obliquely curved row of powerful pyramidal membranelles on the left side, but almost filling the peristomial area. The right margin is sharply cut with a narrow undulating membrane inserted between it and the adoral zone. The macronuclei are eight in number, and, in the vegetative stages,

each possesses a typical nuclear cleft. The micronuclei vary in number from two to six; they are minute and homogeneous. The contractile vacuole is single, spherical, and lies in the center of the body near the dorsal surface. Length of the vegetative forms varies from 140 to 165 μ and the diameter at the center of the body from 11 to 17 μ. The cysts are spherical and the cyst wall smooth with an average diameter of 32.7 μ. (Calkins, 1919, p. 294.)

There are three points in which these descriptions of the supposedly same species do not agree. These are: (1) the length of the organism (Engelmann's 300 μ, the New York variety 158 μ;

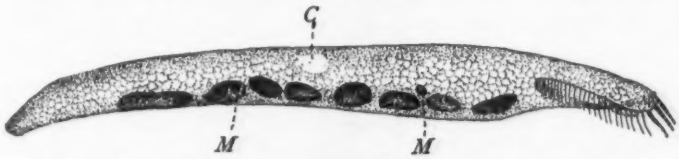


FIG. 1. *Uroleptus mobilis* Engel. New York variety. Cilia not shown. c = contractile vacuole; m = micronucleus. × 650.

(2) the number of macronuclei (Engelmann 6, the New York variety 8); and (3) the position of the contractile vacuole (Engelmann, anterior third of body, the New York variety center of the body). Despite these differences however, it was decided to regard the New York form as an American variety of the European species. While this decision was probably an error in judgment it is too late now to propose a change and I shall refer to it here simply as the New York variety.

Uroleptus Halseyi differs from both *Uroleptus mobilis* and the New York variety although superficially it resembles both (Fig. 2). The body form is constant, plastic, and circular in cross section and about nine times longer than it is broad. The posterior end tapers gradually to form a long sharp tail which is distinctly curved towards the ventral side. The body is much more worm-like than the New York variety and twists and doubles on itself in all directions as it forces its way into zoögloea masses or detritus of different kinds. The average length in fixed preparations is 163.2 μ and the average diameter is 18.7 μ but the organisms are capable of stretching out until the length is sixteen times

FIG. 2. *Uroleptus Halseyi*, n. sp. Cilia not shown. *c* = contractile vacuole; *m* = micronucleus. × 675

the diameter. The size therefore is highly variable and appears to be dependent upon the age of the culture and nature of the medium. The curvature of the tail is much more pronounced than in either of the other forms. The peristome is well marked and more conspicuous than in the New York variety and occupies from one sixth to one seventh of the total length of the body.

The descriptions given above of the three types of *Uroleptus* show very few structural differences of importance so far as the externals are concerned. Internally however, the differences are quite characteristic and well-marked. The cytoplasm of *Uroleptus Halseyi* is filled with iron hæmatoxylin staining granules especially after conjugation. These are interpreted as mitochondria of the protozoan type although in an unbroken cell they do not stain with Janus green. Nor do they stain with neutral red or any other vital dyes. They disappear after long exposure to xylol, turpentine or similar reagents and after a short treatment with acetic acid or other acids. They appear to be therefore, distinctly of a lipoid nature hence if not mitochondria they belong to the same group of cytoplasmic granules.

Other cytoplasmic granules stain vividly with neutral red; still others with methylene blue. The neutral red granules are more or less distributed throughout the endoplasm but are also present in lines running the length of the body on the surface. These are undoubtedly the excretory granules so-called which are characteristic of ciliates generally. The methylene blue granules are also arranged in longitudinal lines but are larger and more numerous than the neutral red granules.

As with the New York variety the number of macronuclei of *Uroleptus Halseyi* is occasionally eight, distributed in a linear series along the ventral half of the cell. This is the usual number in the New York variety, a larger number (nine to twelve) being exceptional. In mass cultures of the new species individuals are rarely found with eight macronuclei the great majority having from ten to sixteen while some individuals have as many as twenty-six. These nuclei take the usual chromatin dyes and give a positive reaction with the Feulgen method.

The micronuclei are very large and quite different from the

	Size.		Macronuclei.		Micronuclei.		Cont. vacuole.	Tail.
	Length.	Diam.	Number.	Size.	Number.	Size.		
U. mobilis	300μ	25μ	6	?	?	?	Anterior third	Inconspicuous
N. Y. Variety	158μ	15.6	8–12	3.8×8.3μ	2–6	2 ×2.5μ	Center—dorsal	Blunt, curved
U. Halseyi	163μ	18.7	8–26	3.2×8.4μ	1–2	2.8×9.3μ	" "	Pointed "

micronuclei of the New York variety. After conjugation the organisms reorganize with two or occasionally three but in many adult individuals only one micronucleus can be found.

Uroleptus Halseyi is not as satisfactory for isolation cultures as the New York variety, but it grows well in mass cultures having a pH of 7.4 to 7.2. Its division and conjugation processes offer some interesting variations from those of the New York variety and these will be taken up in a later paper.

The essential differences in the three types of *Uroleptus* are given in the table on page 64.

Effect of Ultra-Violet Rays on *Uroleptus*.

A Cooper-Hewitt mercury lamp known as the Uviarc was used as the source of ultra-violet rays. The organisms to be exposed were contained in a flat Syracuse dish with a uniform depth of medium of three eighths of an inch. The surface of the medium was fixed at 23 centimeters from the source of light and the exposure was limited to exactly thirty seconds. Permanent preparations of the exposed *Uroleptus* were made ten minutes after exposure, twenty-four hours and forty-eight hours after. Sixty hours after exposure all *Uroleptus* individuals were dead although other species of Protozoa with the same exposure to the ultra-violet rays in the same container, continued to live. The *Uroleptus* material therefore had received a lethal dose but a dose that killed only after two days subsequent to exposure. Material was thus furnished which was suitable for a study of the effects of radiation on the cell constituents.

A culture of *Uroleptus Halseyi* with few dividing forms was used. This culture, four days before was actively dividing. Such actively dividing forms have an average length of 124.3 μ and an average diameter of 22.5 μ. The extremes were 180 x 20 and 120 x 25 μ thus showing a wide variation which is characteristic of actively dividing forms. In ordinary mass cultures the averages of length and breadth are 163.2 x 18.7. These measurements together with similar measurements of individuals killed ten minutes twenty-four hours and forty-eight hours after radiation are shown in the following table:

MEASUREMENTS OF 10 INDIVIDUALS EACH, IN MICRONS.

	Normal.				Exposure to ultra-violet rays.					
	Actively Dividing.		Vegetative.		10 Min. After.		24 Hours After.		48 Hours After.	
	Leng.	Diam.	Leng.	Diam.	Leng.	Diam.	Leng.	Diam.	Leng.	Diam.
	180	20	143	15	132	13	110	16	100	12
	112	26	150	16	150	12	111	16	90	15
	110	25	170	17	145	10	90	14	105	13
	123	24	192	19	145	14	82	13	112	12
	120	19	195	20	111	10	93	14	100	14
	116	22	160	20	142	12	69	20	110	10
	117	23	142	20	148	10	75	13	112	16
	129	27	160	20	110	11	85	14	103	14
	120	13	150	22	130	15	98	12	96	18
	116	26	170	18	122	9	78	13	100	17
Average	124.3	22.5	163.2	18.7	133.5	11.6	89.1	14.5	102.8	14.1

It is evident that one well-marked effect of the ultra-violet rays is a great reduction in size of the organism. This is manifested immediately after radiation as shown by the difference in average size of ten individuals taken at random, from 163.2 x 18.7 to 133.5 x 11.6. The decrease in size continues until at the end of twenty-four hours the average size is only 89.1 x 14.5 μ. Apparently a first effect, therefore is the loss of cell fluids, but there is a partial recovery during the second twenty-four hours, for the average size goes up to 102.8 x 14.1 μ. The organisms however are doomed for by the end of sixty hours after exposure they are all dead. Immediately after exposure they are active and apparently normal.

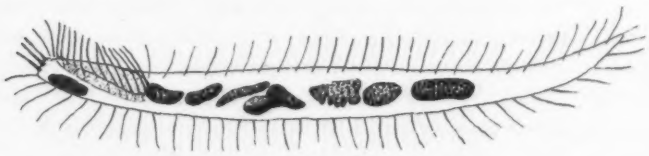


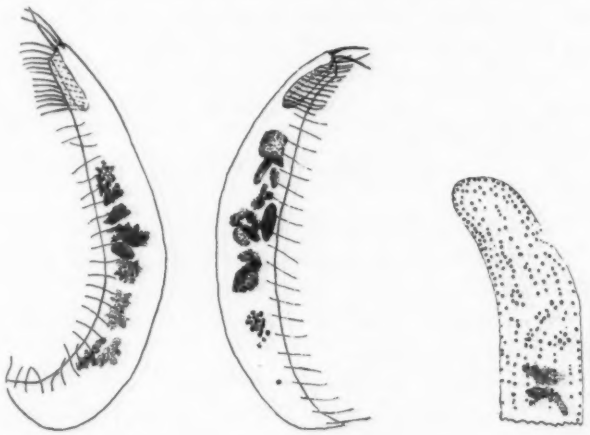
FIG. 3. *Uroleptus Halseyi.* Killed ten minutes after treatment with ultra-violet rays for 30 seconds. ×775.

Other effects of radiation are shown by the intra-cellular structures. The structures which we interpret as mitochondria are

destroyed. They segregate in small granular masses which ultimately turn yellow. This leaves the cytoplasm strikingly clear and the nuclei in consequence are much easier to study.

In material fixed 10 minutes after exposure the nuclei have the normal linear arrangement. Chromatin and X granules are present and a normal picture is given both by the Borrel stain and the Feulgen reaction (Fig. 3).

Twenty-four hours after exposure there is a very different picture. Not only are the cells smaller but they are now uniformly sickle-shape, almost motionless, and have lost their characteristic caudal hooks (Fig. 5). The macronuclei are no longer in linear arrangement but are massed near the middle of the body as they are during the later phases of conjugation. The chromatin granules run together and the nuclei may be broken up into fragments



4 5 6

FIG. 4. *Uroleptus Halseyi.* Killed 48 hours after treatment with ultra-violet rays for 30 seconds. × 775.

FIG. 5. *Uroleptus Halseyi.* Killed 24 hours after treatment with ultra-violet rays for 30 seconds. × 775.

FIG. 6. *Uroleptus Halseyi.* Posterior end of specimen killed 24 hours after treatment with ultra-violet rays for 30 seconds. × 1000.

which may remain together in a semblance of the former nucleus, or they may be dispersed in the cell. In either case there is an

entire disappearance of the nuclear membranes, the nuclei thus becoming emarginate and amorphous. The micronuclei are rarely intact and large portions are usually lacking as though bitten off.

Forty-eight hours after exposure there is no essential change in the picture. Spherical masses of yellow mitochondria (?) are present and are now found mainly in the posterior end of the cell (Fig. 4). Iron hæmatoxylin shows relatively enormous peripheral excretory granules in rows which run the entire length of the cell (Fig. 6). The micronuclei are usually much elongated and in consequence, appear like spindles but they are invariably homogeneous and with no trace of chromosomes or fibers.

The effects of ultra-violet rays on *Uroleptus Halseyi* may be briefly summarized as follows:

1. A reduction in size and a change in shape of the organism.

2. Movement is at first normal but slows down after six to eight hours. At 48 hours the organisms are quiet save for movement of the membranelles; at 60 hours they are dead.

3. Structures usually described as mitochondria disappear immediately after radiation. They collect in granular masses which ultimately turn yellow.

4. The macronuclei undergo granular degeneration and disintegrate.

5. The micronuclei become elongate and disfigured.

6. The characteristic Feulgen reaction is given at all stages indicating that the nucleic acid persists throughout.

7. The peripheral excretory granules are enlarged and apparently are more numerous.

8. An exposure of 30 seconds to ultra-violet rays produces no monsters; this dosage is lethal to *Uroleptus Halseyi,* but not lethal to many other types of Protozoa exposed at the same time.

FURTHER STUDIES UPON THE NEMATOCYSTS OF *MICROSTOMUM CAUDATUM.*

WM. A. KEPNER AND J. W. NUTTYCOMBE,
UNIVERSITY OF VIRGINIA.

The nematocysts of *Microstomum caudatum* are those of hydra which the rhabdocœle has appropriated, after having eaten and digested the polyp. This was first shown by Martin (08), (14). He failed, however, to demonstrate how the *Microstomum* manipulated the nematocysts. Kepner (11) showed that the nematocysts were left naked, by the action of the gastric enzymes, within the enteric lumen (Fig. 1, *a*). The naked nematocysts were then taken up by the epithelial cells of the endoderm as particles of food are taken into food vacuoles by these cells (Fig. 1, *b*); but they are not ejected into the lumen of the enteron again as indigestible portions of the food are from food vacuoles. The nematocysts are then handed over to the mesenchyme by the endodermal cells. Here, for a time, they lie indifferently oriented within lacunæ (Fig. 1, *c*). Eventually a wandering mesenchymal cell appropriates each nematocyst. The mesenchymal cell becomes a cnidophage (Fig. 1, *d*). Meixner (23) published a paper in which he contends that the attending cells of the appropriated nematocysts are not cnidophages but the original cnidoblasts with nematocysts which have been carried all the way through the walls of the enteron and the mesenchyme of *Microstomum lineare* (Mull) to the surface of the rhabdocœle where they retain and discharge the nematocysts. Meixner's observations run quite counter to what we and the other students in this laboratory have repeatedly and without exception observed in *Microstomum caudatum.* We have seen the naked nematocysts lying within the enteron's lumen and also being carried through the endoderm into the mesenchyme in the living specimen as it was held immovable under a coverglass.

If Meixner should be correct in contending that the attending cells, which he and we see in *Microstomum,* be the original cnido-

blasts, the situation is a very remarkable one; for we should then have isolated cells of hydra functioning in coöperation with the tissues of *Microstomum* in the discharge of nematocysts.

The manipulation of the nematocysts on the part of *Microstomum* is not an incidental phenomenon. In the first place the rhabdocœle does not accept hydras primarily for food. *Microstomum* is not like the Æolididæ, in this respect. Evans (22) says, "The Æolididæ all eat cœlenterata" (p. 453). In other words, cœlenterata constitute one of the regular types of food of these molluscs. This fact stands in sharp contrast with *Microstomum's* feeding upon hydra. When a "loaded" *Microstomum* is forced, through inanition, to feed upon hydra, it digests the tissues of the polyp and regurgitates the naked nematocysts after all the protoplasmic parts of the hydra have been digested. On the other hand, a well-fed *Microstomum*—one gorged with food, but lacking nematocysts—will make room for a hydra by ejecting some of its food and at once taking up the polyp. Thus it appears that *Microstomum*, unlike the Æolididæ, does not prey upon a cœlenterate primarily for food but for its nematocysts.

Since *Microstomum* thus seeks primarily the nematocysts of hydra and not its tissues, it must have some definite demand for these defensive and offensive structures of hydra. Kepner and Barker (24) have recorded much evidence of *Microstomum* using the nematocysts of hydra which it has appropriated.

The appropriation and use of hydra's nematocysts is, therefore, quite as definitely an instinct as is the appropriation of materials for and the building therewith of a nest on the part of a bird. Moreover, this is a complex instinct in that it involves the selective faculties of the *Microstomum* as a whole when it takes hydra, and of the endodermal cells when they take up and hand over to the mesenchyme only nematocysts and do not lay hold of other solid bodies and deliver them to the mesenchyme. The complexity of this instinct is further shown in that the cnidophages (or, if Meixner be correct, the cnidoblasts) carry the nematocysts to the surface in such a manner that the latter are uniformly distributed throughout the surface of the flatworm. Here the cnidophages act coöperatively. When once the nematocysts have been carried to the surface, the cnidophages function independently. When a

"loaded" *Microstomum* is locally disturbed, only the nematocyst or nematocysts in the immediate vicinity of the disturbing factor will be shuttled to and fro in a threatening manner. If the disturbance be maintained, only the local nematocysts will be discharged.

Thus it is seen that in *Microstomum's* handling of the nematocysts of hydra we have a complex instinct. The factors for instincts are usually transmitted from generation to generation by the gametes. *Microstomum,* under laboratory conditions, seldom elaborates gametes and zygotes. In its natural habitat, one usually encounters them propagating asexually, but in the autumn zygotes appear.

In *Microstomum,* therefore, we recognized an opportunity to test the possibility of transmitting factors for an instinct, not through gametes, but, through somatic cells as the animal propagates asexually through fission, if a method for rearing *Microstomum* could be discovered.

Three years of casual effort netted only failure to rear *Microstomum* in the laboratory. Light and temperature conditions were variously controlled. The water in the culture dish was exchanged each day for water that had just been taken from the pond in which *Microstoma* lived. But with all no positive results were secured. Eventually it dawned upon us that perhaps the specimens needed sediment within which to be anchored, as it were, at rest. When we added a little dried sediment collected from the pond, we found that the animals no longer kept on the move, but spent much of the time lying quietly within the sediment and that thereafter we had little difficulty in carrying them as far as into the twenty-sixth asexual generation.

Having succeeded in finding a method of culturing them, we next attempted to determine whether the persistence of the complex instinct, involved in the handling of nematocysts by *Microstomum,* would be carried from asexual generation to asexual generation without experience with hydras.

This attempt was made by selecting wild individuals, that lacked nematocysts, from their natural habitat and establishing clones with them.

Clone "A′" was established April 30, 1926, from a wild indi-

vidual that lacked nematocysts. By May 19, the seventh asexual generation had made its appearance. One of the two individuals of this generation was placed with a brown hydra. This hydra was eaten and its nematocysts appropriated. Similar results were obtained for an individual of the tenth asexual generation of this clone. A second clone "A" we succeeded in carrying into the twenty-sixth asexual generation before it died. One individual of the twenty-third asexual generation was placed with an hydra, which was later, in part, eaten and its nematocysts appropriated. Thus we found that *Microstoma* removed from experience with hydra by six, nine or even twenty-two asexual generations will accept and appropriate nematocysts from hydra upon which they have fed.

Having established the fact that the instinct involved in the manipulation of hydra's nematocysts persists through a score of asexual generations, we next attempted to break the persistence of the instinct by bringing about the intervention of regeneration on the part of *Microstomum* as well as the intervention of asexual generations which lacked experience with hydra.

A specimen was, therefore, taken from the sixteenth asexual generation of clone "A," to be experimented upon, on November 20, 1926. This specimen was held quiet in a shallow layer of water and decapitated along line *a*, Fig. 2. This involved the removal of mouth, pharynx and dorsal ganglia (the only ganglia present in *Microstomum's* nervous system.) There had been no evident fission-plane in this specimen. However, following the cutting, much of the enteron oozed out from the cut end, which was followed by an abstriction along line *c*, Fig. 2. This resulted in two incomplete zoöids *A* and *B*. It is possible that the rudiments of dorsal ganglia might have been present in zoöid *B*. Zoöid *A* was so much reduced because of the exudation of tissues that it was not a promising specimen. It was rejected and zoöid *B* was cared for. Four days later (November 24) zoöid *B* had formed a cephalic region and accepted tadpole liver. After it had fed, a large green hydra was placed with it. Within five minutes three of us, in this laboratory, saw the *Microstomum* feeding upon hydra. On November 26, the specimen displayed many nematocysts within its mesenchyme and a few at the surface. On No-

vember 27, many nematocysts were distributed uniformly at the surface of *Microstomum*. Thus it was seen that the posterior zoöid, which was induced to abstrict from the parent prematurely and thus lacked a fully organized complement of anterior organs, accepted and appropriated, after regeneration of an anterior region, the nematocysts of hydra, despite the fact that it had been removed from experience with hydra at least fifteen asexual generations.

We were also prompted to make a similar attempt by using an incomplete anterior zoöid. Accordingly, from the seventeenth generation of the same clone "A," an individual was chosen on November 23 that showed no fission plane when examined under the 4 mm. objective and 10 × eyepiece. The specimen was cut, as it lay in a shallow sheet of water, along the line indicated by *a* in Fig. 3. In reaction to this operation, this specimen, too, divided along the plane indicated by *b* in Fig. 3. The posterior zoöid was rejected. The anterior zoöid had now lost its mouth, pharynx, cephalic ganglia and caudal region. It is possible that near its mid-transverse plane there had been the merest rudiments of cephalic ganglia. It is certain, however, that there were no cephalic ganglia at its anterior wounded end. The recorded history of this zoöid is as follows:

On November 25, two days after the operation, it was not able to swim in a straight course; only after repeated attempts to feed it, was it induced to eat some tadpole liver. On November 27, it was not, as yet, swimming along a straight path. In the meantime, not only had the anterior and posterior structures been fairly well regenerated, but a conspicuous fission-plane had formed in the zoöid's mid-region. The specimen now attempted to feed upon tadpole brain. Its reaction was quite awkward. In trying to assist it, the posterior zoöid was torn from the specimen. This posterior zoöid was discarded. On November 29, the specimen was no longer awkward in its swimming and fed upon tadpole liver. It was then placed with a seven-tentacled green hydra December 4. On December 6, the *Microstomum* had many nematocysts distributed over its surface and the hydra had lost one and part of another of its tentacles. The *Microstomum* then accepted a large meal of tadpole liver. Thus it is seen that

the anterior half of a prematurely divided *Microstomum,* from which the mouth, pharynx, and cephalic ganglia had been removed, will accept and appropriate the nematocysts of hydra, even though this partly regenerated zoöid had been removed from experience with hydras by at least sixteen asexual generations.

Meixner ('23) was unable to observe *Microstomum lineare* discharge any of its nematocysts. He further cited the absence of cnidocils or releasing mechanisms associated with the nematocysts of *Microstomum.* He, therefore, challenged the senior author's suggestion that *Microstomum caudatum* uses its nematocysts. Meixner had not seen the records of Kepner and Barker ('24) which were yet in editors' hands. These authors record observations of *Microstomum* discharging at and wounding other animals with its appropriated nematocysts.

Meixner's paper has suggested to us that it might be well to determine whether *Microstomum,* when removed from experience with hydra by ten or more asexual generations, could use the nematocysts that it had appropriated from hydra. Accordingly such specimens were issued to four graduate students.

Mrs. J. S. Carter reports that she "was successful only in so far that I saw them eat hydra and retain the green coloration for at least twenty-four hours, but the *Microstomums* died before I completed the experiment."

Mr. Paul R. Burch also gave a negative report as follows: "A *Microstomum* ten generations removed from the use of hydra as food was received February 28, 1927, and placed with hydra March 2d. The *Microstomum* showed nematocysts the next day, 22.5 hours later. In the meantime it had divided. One of the daughter *Microstoma* was placed in a hanging drop of water in a moist chamber with two *Stenostoma* and observed almost continuously for three hours. During a great part of this time one or the other of the *Stenostoma* was in contact with the *Microstomum* and pushing against it. Time and time again one or the other *Stenostomum* forced its way between the *Microstomum* and a piece of detritus or between *Microstomum* and the other *Stenostomum,* yet the *Microstomum* did not discharge a single nematocyst during this time. It was noticed, however, that at least one third of the nematocysts were not yet oriented for discharge."

Mr. Wesley Fry obtained a *Microstomum*, which was fifteen generations removed from experience with hydra. The animal had no nematocysts. He reported: "I fed this specimen a green hydra and on Saturday, March 5, the animal had appropriated the nematocysts of the hydra. They appeared scattered throughout the ectoderm of the *Microstomum*. I placed this animal in contact with a *Stenostomum* in a hanging drop and saw it discharge nematocysts at the *Stenostomum*."

Mrs. S. B. Grimes used some *Microstoma* which were fifteen generations removed from contact with hydra and were, therefore, quite free from nematocysts. He reported: "I fed them green hydras and in the course of twenty-four to forty-eight hours the nematocysts became oriented in the ectoderm of the *Microstoma*. I then placed one of these 'loaded' *Microstoma* in a hanging drop along with a small oligochæte and watched the reaction. The setæ of the worm agitated the *Microstomum* so that it oscillated its nematocysts as though to discharge them. The setæ continued to irritate the flatworm and several nematocysts were thrown out. The threads of some of them pierced the body-wall of the oligochæte and partly paralyzed the worm. Dr. B. D. Reynolds and Mr. J. R. Mundie each checked up on what I had seen."

Thus it was demonstrated by two of these observations that a *Microstomum*, removed by as many as fifteen asexual generations from experience with hydra, can not only appropriate the nematocysts of the latter but can actually use these nematocysts and discharge them into the bodies of offending neighbors.

This persistence of factors for instinct of *Microstomum* would seem to be the outcome of the potentialities of the various tissues and cells of the body. For, in the first place, gametes and zygotes have not been involved in our experiments. The persisting of factors for instinct, therefore, could not have resided in the germplasm in this case. Secondly, the neuroplasm has not been involved in transmitting this instinct. Ritter and Congdon ('00) claim that in *Stenostomum* there is a migration of neurones from the anterior zoöid into the incipient posterior zoöid. Child ('02) has not been able to observe such migration. The senior author has made frequent observations upon the histogenesis involved

in fission of *Microstomum*. No migration of neurones has been observed by him. On the contrary, the ganglia arise locally as internal epidermal proliferations. The neurones for each generation, therefore, arise independently from the epidermis. Moreover, the conduct of *Microstomum* in manipulating these nematocysts precludes neural control; for the cnidophages carry the nematocysts to the surface as free cells operating quite as independently of the central nervous system's direct control as do the phagocytes of one's own body. And when once they have established themselves at the surface of the *Microstomum's* body they respond to threatening stimuli independently. In this respect they fall under the class of cells that Parker ('19) refers to as "independent effectors" (pp. 83–84). If, on the other hand, the cells attending the nematocysts in *Microstomum* be not cnidophages but cnidoblasts of hydra (as Meixner ('23) maintains), they are quite foreign to the neuroplasm of *Microstomum*.

The persistence of factors for an instinct, independent of the nervous system and so complex as that concerned with the collecting and manipulation of the nematocysts of hydra on the part of *Microstomum* is contrary to the prevailing ideas that the factors for instincts reside in the neuroplasm of the individual and are transmitted through the germplasm. That the factors, which determine the presence of instincts, are considered to lie within the neuroplasma is indicated by two references we have chosen to make. Many others might have been selected. D. Forel, in reviewing the instinctive conduct of ants, calls attention to the fact that the ant's brain surpasses in relative volume and in complication of structure that of all other insects. Again, Charles Darwin ('74) says: "It is certain that there may be extraordinary activity with an extremely small absolute mass of nervous matter; thus the wonderfully diversified instincts, mental powers, and affections of ants are notorious, yet their cerebral ganglia are not so large as the quarter of a small pin's head. Under this point of view, the brain of an ant is one of the most marvelous atoms in the world, perhaps more so than that of man." These two references represent the attitude usually taken concerning the relation between conduct and neural structure.

But in this case, the instinct, involved in handling the appropri-

ated nematocysts, can not be said to be controlled by the central nervous system of *Microstomum*. The control of the manipulation of the nematocysts through hormones is suggested, but it seems better to consider this manipulation to be the outcome of the potentialities of all of the cells of *Microstomum's* body.

There are not many examples on record of factors for an instinct persisting through generations that have not put into operation this instinct. C. R. Griffith ('19) found that white rats, that had been members of the tenth generation of inbreeding, showed a pronounced instinctive fear of a cat, though the previous nine generations had not experienced the presence of a cat. But Griffith says that this reaction on the part of the rats "may not be specifically related to the situation. . . . Any other new stimulus may arouse such reactions, the necessary component of total perception being just the unfamiliarity or strangeness and not the specific feline odor" (p. 167).

In the conduct of *Microstomum*, dealing with the nematocysts of hydra, there is a reaction that is quite definitely specific. Hence we have a clear case of an instinct being transmitted through as many as twenty-two asexual generations though it had not been exercised in twenty-one previous ones.

Summary.

Microstomum caudatum, after being kept from experiences with hydra for twenty-two asexual generations, will feed upon hydra and appropriate the nematocysts. The removal of all of the ganglia and the intervention of extensive regeneration in *M. caudatum*, sixteen asexual generations removed from hydra, will not prevent the transmitting of factors for the instinct that deals with the manipulation of the nematocysts of hydra by *Microstomum*. The appropriated nematocysts have been *used* by an individual as much as fifteen asexual generations removed from hydra.

The instinct is in this case, therefore, not resident in the neuroplasm and the factor determining its presence is transmitted from generation to generation by the soma and not by germplasm.

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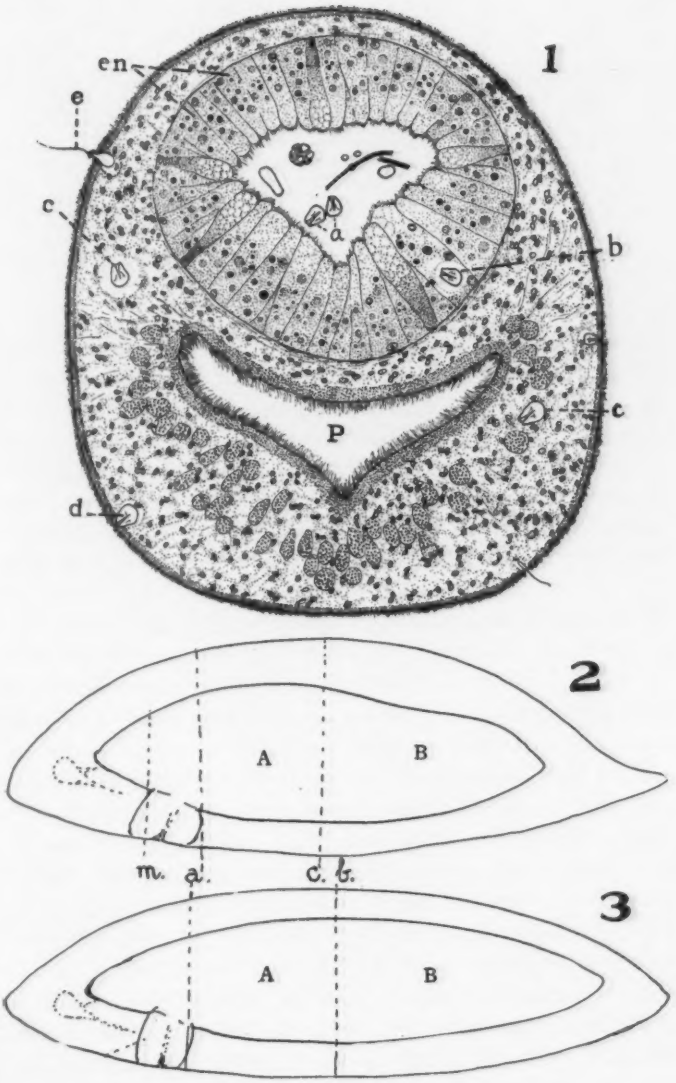
EXPLANATION OF FIGURES.

FIG. 1. Transverse section of *M. caudatum* taken through plane shown by *m* in Fig. 2. *a*, naked nematocysts of hydra lying within lumen of enteron; *b*, naked nematocyst within a vacuole of an endodermal cell; *c* and *c*, naked nematocysts within lacunæ of mesenchyme; *d*, nematocyst oriented and accompanied by a cnidophage, the nucleus of which is shown at base of nematocyst; *e*, partly discharged nematocyst; *en*, endoderm of enteron; *P*, pharynx. From Kepner ('25).

FIG. 2. Specimen fifteen asexual generations removed from experience with hydra. *a*, plane along which body was severed; *c*, plane along which body abstricted after the anterior end had been cut away, thus forming two incomplete but free zoöids (*A* and *B*); *m*, plane of transverse section shown in Fig. 1.

FIG. 3. Specimen sixteen asexual generations removed from hydra. *a*, plane along which specimen was severed; *b*, plane along which body abstricted after the anterior end had been cut away, thus forming two incomplete but free zoöids (*A* and *B*).



W. A. KEPNER AND J. W. NUTTYCOMBE.

STUDIES ON THE PHYSIOLOGY OF THE EUGLENOID FLAGELLATES. I. THE RELATION OF THE DENSITY OF POPULATION TO THE GROWTH RATE OF *EUGLENA*.

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INTRODUCTION.

The relation of volume of culture medium to the rate of growth of ciliates has been investigated by a number of workers during the last two decades, but up to the time of this investigation no similar experiments on the green flagellates had been attempted. The object of the present investigation, therefore, was to determine whether or not such a relationship exists in growth of *Euglena*, and also whether or not there is evidence for an "allelocatalytic effect," such as described by Robertson (1921*b*, 1922) for ciliates.

For this series of experiments *Euglena* sp. was chosen for its ability to grow rapidly in "autotrophic" media. Also, in such a medium the food supply of an organism is much less affected by bacterial activities than if a large amount of organic material were present. The first experiments were carried out in the same manner as were previous isolation experiments with ciliates (Woodruff, 1911, 1913; Robertson, 1921*a*, *b*, 1922; Cutler and Crump, 1923*a*, *b*, 1924, 1925; Greenleaf, 1926; Myers, 1927; Yocom, 1928). This method was discarded later in favor of a mass method in which variations in the concentration of organisms were substituted for variations in volume of the medium.

This investigation was carried out at the suggestion of Doctor R. P. Hall, and the writer wishes to express his gratitude for suggestions and aid in the preparation of the present paper. The writer also wishes to thank Professor Asa C. Chandler for the use of the Biological Laboratory of the Rice Institute during the summer of 1928.

Historical Survey.

The earliest observations on the relation of volume of culture medium and division rate were those of Woodruff (1911) on two species of *Paramecium* (*P. caudatum* and *P. aurelia*), which showed a more rapid division rate in larger than in smaller volumes of the same culture medium. Woodruff suggested that this was due to a more rapid accumulation of waste products in cultures of smaller volume, and in later observations (Woodruff, 1913) he showed that waste products of any one species (of *Paramecium, Stylonychia, Pleurotricha*) actually depressed the division rate of the same species, although apparently without effect on other species.

The next investigations in this field were carried out by Robertson (1921*a*, *b*, 1922, 1924*b*, *c*) who found that when two ciliates were isolated into the same drop of culture medium the division rate was higher (sometimes sixteen times as great) than if a single cell were isolated into a drop of the same size. This difference in division rate is, according to Robertson, due to the mutual contiguity of the cells, and has been designated by him as an "allelocatalytic effect," caused by a growth-catalysing substance liberated from the nucleus into the cytoplasm and thence into the surrounding medium during nuclear division. This theoretical catalyst is called by Robertson an "autocatalyst of growth." The amount of this catalyst present in isolation cultures would depend upon the number of cell divisions that had taken place; therefore, the culture with the greater number of dividing cells would have a greater concentration of catalyst, and the division rate in these cultures should be correspondingly greater.

Cutler and Crump (1923*a*, *b*) found no evidence for the occurrence of allelocatalysis in cultures of *Colpidium* with 1, 2, 3, and 4 initial organisms in volumes that varied from 0.5 to 8.5 cubic millimeters, or in mass cultures with concentrations of 100–8,000 organisms per cc. They pointed out that their results as well as those of Robertson, might be explained by the presence of a larger food supply in the cultures showing a higher division rate.

Greenleaf (1924, 1926), in a large number of experiments in which the volume of medium was varied from two to forty drops,

found that two species of *Paramecium* (*P. aurelia, P. caudatum*) and *Pleurotricha lanceolata* showed a higher division rate in larger volumes of medium, and also that in cultures of *Stylonychia pustulata* the division rate of a single animal was greater than that of either of a pair of animals when introduced into the same volume of medium.

Calkins (1926) made a sixty-day test of *Uroleptus mobilis* with one, two, three, and four individuals per drop. The cultures with one initial organism showed a higher division rate than those with two organisms, and those with two organisms a higher division rate than those with three and four.

Myers (1927), in a long series of experiments, found that the division rate of *Paramecium* decreased with the density of population, and that a certain minimum volume (0.2–0.4 cc., or 4–8 drops) of "flourishing" hay infusion was necessary for a single *Paramecium* to multiply at its maximum rate.

Yocom (1928) found that in *Oxytricha* four-drop cultures had a 14 per cent. higher division rate than ten-drop cultures. In each series of experiments, however, there were some cases in which the division rates of the ten-drop cultures were equal to or higher than those of the four-drop cultures. Only by averaging a number of cases could he show an appreciable difference. He accredits this difference to the earlier attainment of a high concentration of autocatalyst in the cultures of smaller volume.

Petersen (1929) has found that within certain limits a higher division rate of *Paramecium* is present in cultures of smaller volume and in cultures containing a larger number of ciliates. Some of her results, however, tend to contradict Robertson's theory.

In reviewing the work on volume-division rate relations in Protozoa one finds that Robertson's theory is supported only by the work of Yocom and by some of the results of Petersen. On the other hand, the experiments of Woodruff, Cutler and Crump, Greenleaf, Calkins, Myers, and some of the results of Petersen, furnish evidence which contradicts the theory of an autocatalyst.

Material and Methods.

Early in the summer of 1928 water was collected from several ponds in the vicinity of Houston, Texas, including that known

as the "Biology Pond" of the Rice Institute. This was placed in aquarium-jars and gave rise to mixed euglenoid cultures. An autotrophic medium similar to those used by Mainx (1928) and Günther (1928) was prepared as follows:

NH_4NO_3	10 gms.
KH_2PO_4	5 gms.
$MgSO_4$	1 gm.
H_2O (distilled)	1000 cc.

This stock solution was diluted ten times, brought to pH 7.0 with Na_2Co_3, and then boiled before being used. Culture material was centrifuged, the supernatant liquid poured off, and the synthetic medium added. By repeated centrifugings and washings over several days the organic content was reduced to a minimum. Some of the *Euglena* present continued to grow and gave rise to a mass culture in the autotophic medium. By means of a mouth-controlled suction pipette single specimens of *Euglena* sp. were isolated into fresh medium in depression slides. All *Euglena* used in subsequent experiments were descendants of a single specimen isolated at this time. The depression slides were kept on racks in dessicators and large petri dishes half filled with water.

In order to determine the relation, if any, of volume of culture medium to division rate, sister cells were isolated into different amounts of medium. On account of the small size of the flagellates, the most practicable volumes for such cultures ranged from two to six drops. In all, twenty-two pairs were started at various times. In every case a flagellate was chosen at random from the mass culture derived from a single *Euglena*. Each organism was isolated into a depression slide. After the first division the daughter cells were washed twice in three drops of medium, then placed in separate depressions on another slide and immediately covered with fresh medium—one with two drops, the other with six. For each pair of cultures, counts were made daily and the numbers present recorded.

At the end of ten days, three pairs of cultures showed from two to three and a half times as many organisms in the cultures of smaller volume. In one pair, however, three times as many were present in the culture of larger volume. In another instance the numbers were about equal. The rest of the cultures were dis-

carded on account of excessive evaporation. Such results are by no means conclusive, since they indicate a marked variability in the division rate of this flagellate.

The isolation method in this case, therefore, seemed to be unreliable, and was abandoned for the following reasons: (1) The mortality rate was high, due to the evaporation of the medium. (2) Evaporation increased the salt concentration, and consequently the osmotic pressure of the medium. (3) The practicable range of variation in volume was limited (two to six drops). (4) The results obtained were contradictory. In many cases, synchronous division did not occur in pure lines derived from sister cells left undisturbed in the same depression slide. (5) An extremely large number of experiments would be necessary in order to obtain growth curves that would approach accuracy. (6) Bacterial counts could not be made for such cultures without greatly altering the volume of the medium. In order to avoid as many as possible of these difficulties the mass culture method was adopted.

The same pure line used in isolation cultures was grown in mass cultures in the autotrophic medium already described. By repeated centrifuging and washing, cultures with low bacterial counts were obtained. These were concentrated in centrifuge tubes, and various dilutions were made. In 18×40 mm. test tubes, 20 cc. of each dilution was kept at room temperature under a constant source of light, consisting of a battery of three 100-watt globes (frosted on the inside). In series I., II., III., and IV., the cultures were protected from the heat by a wind tunnel with top and bottom of thin plate glass. An eight-inch fan removed practically all of the surplus heat. The battery of lights was above the tunnel and the cultures below. Mirrors were placed both above the lights and below the cultures in order to insure as uniform a distribution of light as possible. The number of organisms per cc. was counted at the beginning of the experiment and at convenient intervals thereafter. Bacterial counts were made by plating according to the standard method used in water analysis.

Bacterial counts were made on each dilution at the beginning and at the end of each series and in some cases at the end of thirty-six hours also. Initial counts were always low, ranging from 200 to 1,500, and in a few cases to 3,000 per cc. Thirty-six hour

counts were much higher, ranging from 20,000 to 50,000 per cc. Final counts ranged from 30,000 to several hundred thousand per cc. Since the effect of bacterial products on the rate of division of *Euglena* is unknown in this case the significance of the counts can not be stated. It is worth noting, however, that the larger number of bacteria after the first day did not result in an increased division rate. On the contrary, the division rate in cultures of *Euglena* sp. decreased continuously after the first few divisions, thus indicating that their growth was either hindered or else unaffected by the increase in number of bacteria. If bacteria hindered the division rate, this might possibly be effected in one or both of two ways—by utilizing and thereby decreasing the food supply of the euglenoids, or by excreting products of an injurious nature into the medium. However, there is no evidence supporting either possibility, or indicating that the bacteria affected the division rate of the flagellates in any way.

In series V. and VI., Carrel tissue culture flasks were used instead of test tubes. The Carrel flasks assured a much more uniform distribution of light than did the test tubes. The flasks were only half filled in order to allow air circulation. They were suspended in a wire rack and partially immersed in a Freas Water Thermostat (of the small type, accurate to .1° C. for long periods of time) at 27° C.

A Sedgwick-Rafter counting chamber of 0.5 cc. capacity and a Whipple micrometer were used for counting. The method of counting was, in general, that described by Whipple (1927) for counting plankton. Each tube or flask was sampled twice, and usually seventy-two 1 mm. squares were counted and averaged for each sample. If the counts for the two samples differed as much as ten per cent., a third count was made. These three counts were then averaged, and the result was taken as the count for the tube or flask. When duplicate flasks were used each flask was sampled twice, and a count was made for each. The counts for the two flasks were then averaged to find the final count for the dilution in question. In determining the initial concentrations, the average count of five samples was taken in order to insure as great accuracy as practicable. Pipettes for filling the counting chamber were washed thoroughly, autoclaved or boiled, and then dried before being used.

In all cases the concentration of flagellates has been expressed as thousands per cc. or decimal fractions thereof. For example, a concentration of 540 individuals per cc. is expressed as .54 thousand per cc., or merely as .54. No large series of counts was undertaken to determine the exact percentage error, but it is believed that the error is rather low. For example, one set of initial counts was as follows: .87, .90, .87, .91, .92, with an average of .894. Assuming that the flasks were well shaken before sampling, the standard error of such a system of counting is plus or minus the square root of the total number counted (Fisher, 1925, p. 59). In the set of initial counts mentioned above this would be $\sqrt{321}$ or $\pm$ 18, giving as the total corrected number 321 $\pm$ 18, or a concentration of .894 $\pm$ .047. The probable error is always .67499 times the standard error, and in this case is .67499 $\times \pm$ .047, or $\pm$ .0317, making the concentration .894 $\pm$ .032. This is a probable error of 3.52%. However, since the greatest deviation of the individual counts from the mean is .026 in the series mentioned, it is fairly certain that the error is very much less than the standard and is well within the probable error. Concentrations of less than .5 were not counted because of the large number of squares that would have to be counted in order to insure a fair degree of accuracy.

It may seem that four hundred organisms per cubic centimeter is a high concentration, and that from the beginning the cultures might suffer from crowding. However, if the total volume of the organisms is compared to the volume of the medium, it is seen that this is not the case. Taking the radius of a rounded organism as approximately eleven microns, we may calculate its volume to be 4.189×11^3, or approximately 5,575 cubic microns. Since one cubic centimeter is equal to 10^{12} cubic microns, the ratio of the volume of 450 *Euglena* to that of their containing fluid is $\frac{450 \times 5{,}575}{10^{12}}$, $\frac{2{,}608{,}750}{10^{12}}$, or approximately 1 : 383,300. This means that in the most concentrated cultures of all except the first series of experiments, the initial volume of the flagellates was approximately 1/383,300 that of their surrounding medium, while in the most dilute cultures the volume ratio of organism to medium was 1 : 3,833,000.

Experimental Results.

Series I.

On October 14 the organisms were washed and centrifuged six times. On October 15 they were centrifuged five times before the experiment was started. Since the centrifuge tubes were filled to approximately 18 cc. and about 17.75 cc. of supernatant liquid was poured off each time, the concentration of any catalyst of growth, if present, was reduced to approximately 1/72 $\left(\frac{18-17.75}{18}\right)$ of its previous concentration with each washing. Two consecutive washings would reduce the autocatalyst to approximately 1/5,000 of its original concentration; five washings to 1/1,900,000,000. The organisms, after washing, were concentrated by centrifuging and then counted. The concentration was found to be 39.2 thousand per cc. Various numbers of organisms were placed in five culture tubes, which were then filled with medium to a volume of 20 cc. The concentration was then determined for each tube. The initial count in tube number 1 was 0.42; in tube 2, 0.82; tube 3, 1.9; tube 4, 5.0; tube 5, 9.5.

Counts were made subsequently at intervals of 24–36 hours. The average counts were accurately plotted against time, as shown in figure 1. In Fig. 2 the same curves are repeated, but in this case x/x_0 (where x represents the initial number, *e.g.*, .42 in tube number 1) is plotted against time; in other words the curves shown in Fig. 1 are all reduced to unity in Fig. 2. It is readily seen that, in general, the increase in number per initial organism was much greater in the more diluted cultures (Fig. 2). An objection to the concentration used is readily seen in the fact that the more concentrated cultures were initially quite crowded; hence they were probably restricted sooner and more severely by limiting factors than were the more diluted ones. Therefore, other series of experiments were started in which all dilutions were higher than that of culture 1 of series I. It was hoped that these would yield a set of curves in which the limiting factors would be negligible for the first few days. The results of such experiments are described in series II., III., IV., V., and VI.

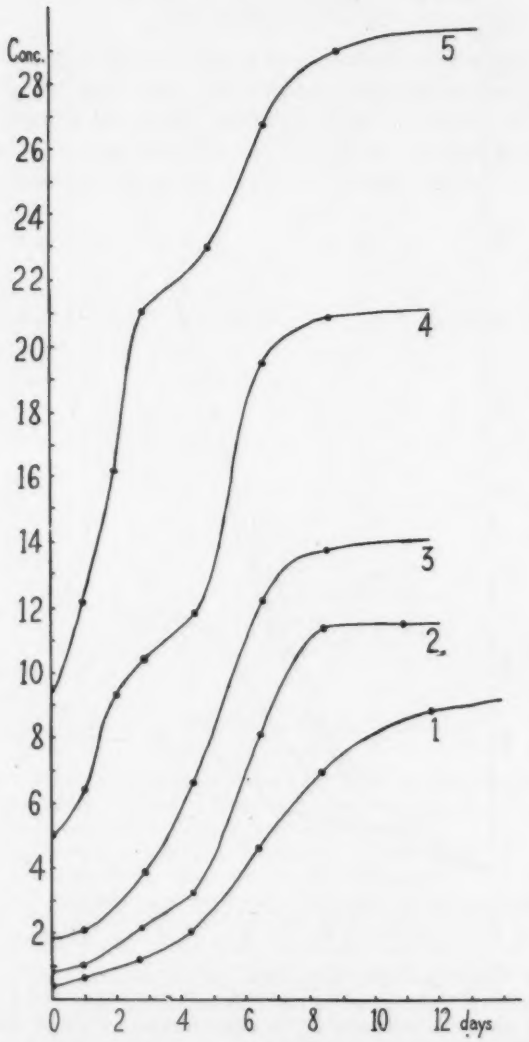

FIG. 1. Graph showing concentration of organisms (in thousands per cc.) plotted against time for series I. Curves 1, 2, 3, etc., represent the numbers present in tubes 1, 2, 3, etc., respectively. Dots represent observed points.

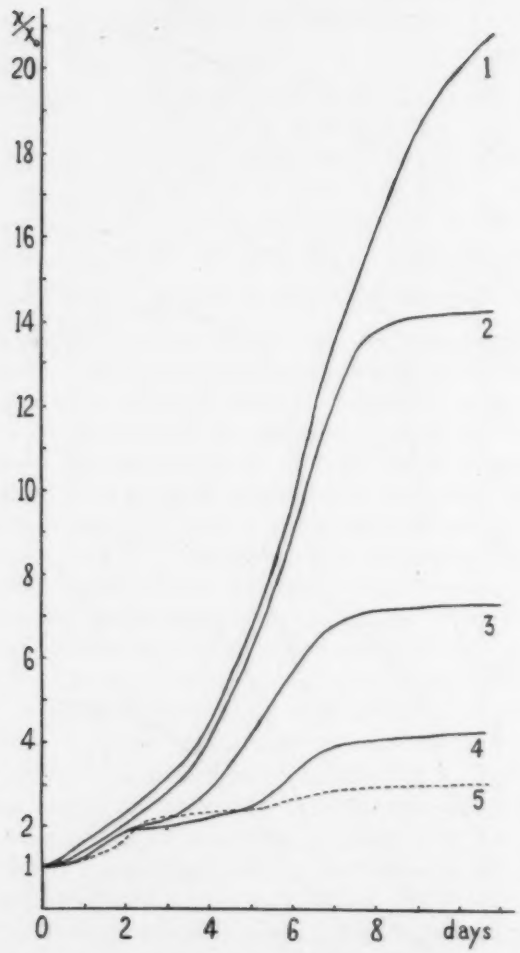

FIG. 2. Graph showing x/x_0 (x_0 being the number at any time t; x_0 the initial number) of the curves of Fig. 1 plotted against time. Note that the order of the curves is reversed. The most concentrated culture (curve 5) shows the smallest increase (with a ratio of 3:1), while the most dilute (curve 1) shows the largest increase (ratio of 21:1). The others show intermediate ratios varying in reverse order with their original concentrations. These curves thus indicate higher rates of division in the less concentrated cultures.

Series II.

The organisms were centrifuged a number of times over several days, and three times just before the experiment was started. Three dilutions were used, and they were maintained in duplicate, the tubes being designated as 1*A*, 1*B*, etc., respectively. One concentration was made and counted. It was found to be .555.

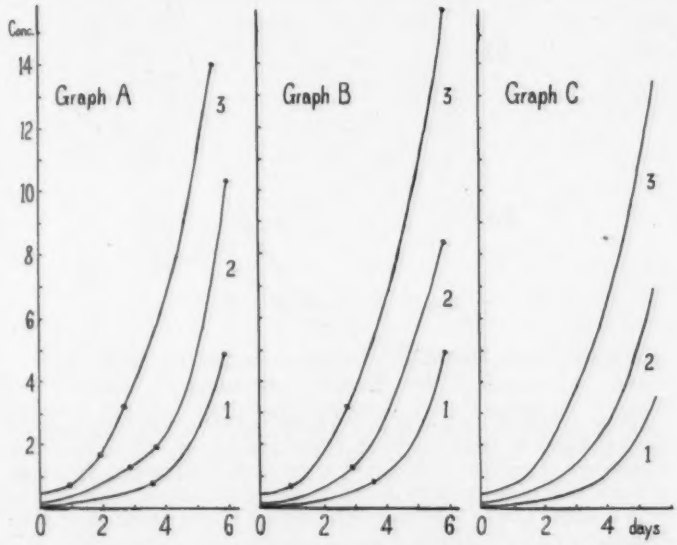

FIG. 3. Graphs showing concentrations for series II. plotted against time.
Graph *A*.—Curves 1, 2, and 3 show numbers in cultures 1*A*, 2*A*, and 3*A*, respectively. Dots indicate observed points.
Graph *B*—Curves 1, 2, and 3 show numbers in cultures 1*B*, 2*B*, and 3*B*, respectively. Dots indicate observed points.
Graph *C*—A combination of graphs *A* and *B*, obtained by averaging corresponding values for the curve of the two preceding graphs.

The fact that graphs *A* and *B* are very similar and that the values of x for any dilution at any given time do not vary much in the two sets of cultures is a demonstration of the efficiency of the method used. Initial numbers were the same for the corresponding curves, conditions were the same for the two sets of cultures, and the resulting curves are very similar.

Twenty cc. of this was placed in tubes 3*A* and 3*B*. Part of the remainder was diluted one to three with sterile medium to make a concentration of .14. Twenty cc. of this was placed in tubes 2*A*

and 2*B*. The remainder was diluted one to nine to make a concentration of .055, of which 20 cc. was placed in tubes 1*A* and 1*B*. The results are shown in Fig. 3. Laboratory conditions prohibited the continuance of the experiment more than six days. Fig. 4

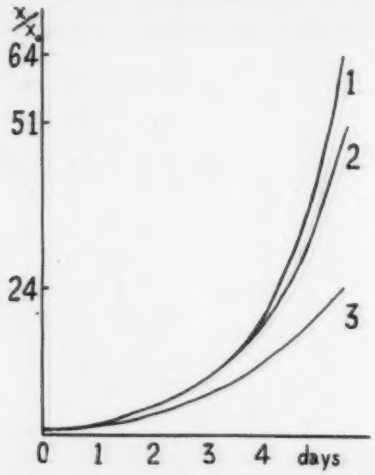

FIG. 4. Graph showing x/x_0 (x being the number at any time t; x_0, the initial number) of graph *C*, Fig. 3, plotted against time. Note the higher relative rate of increase (ratio 64:1) in the more dilute culture represented by curve 1, as compared with the most concentrated culture represented by curve 3 (ratio 24:1). This is directly opposed to any theory of an autocatalyst.

shows x/x_0 of the preceding curve (Fig. 3) plotted against time. The higher division rates of the more dilute cultures are obvious, especially after the first few days when the accumulative differences become quite large.

Series III.

The organisms were centrifuged at least a dozen times during several days and three times just before the beginning of the series. The flagellates were counted. The concentration in tubes 3*A* and 3*B* was .35. The dilution was 1 to 3 for tubes 2*A* and 2*B* and 1 to 9 for tubes 1*A* and 1*B*, as in the previous series. At the end of the first 24 hours (Fig. 5) the count for the most concentrated cultures was .73, and at the end of 48 hours was 1.32, and at the

end of 72 hours was 2.07 as shown in Fig. 5. At the end of 72 hours the count for the second dilution (tubes 2*A* and 2*B*) was .51. Due to an accident no count for the highest dilution could be obtained. For this reason the series was discontinued. The initial concentration in tubes 2*A* and 2*B* was ¼ of that in tubes 3*A* and 3*B*. This ratio was still maintained at the end of the third day when counts were .51 and 2.07.

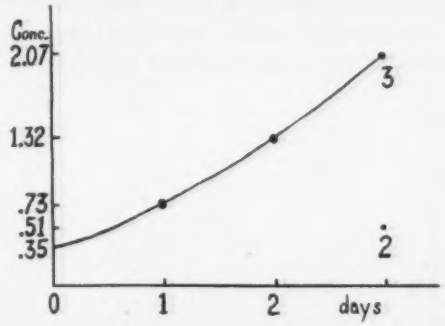

FIG. 5. Graphs showing concentrations in series III. plotted against time. Due to an accident data for one curve and for only one point on another curve was obtained. These were dilutions 3 and 2. The ratio of the initial concentrations was 4:1, and the ratio of their final concentrations at the end of the third day was 2.07:.51—practically the same as the original. This experiment shows no difference in division rate in the two sets of cultures and is opposed to the theory of an autocatalyst.

Series IV.

The flagellates were centrifuged several times, dilutions were made exactly as in series II. and III., and cultures were maintained in duplicate. The initial count for the most concentrated cultures (dilution 3) was .894. The concentration of dilution 2 was .233; that of dilution 1 was .089. The final counts at the end of seven days on the three sets of cultures (six tubes) showed no large difference of growth rate. The final counts were 0.61, 1.50, and 6.68 for dilutions 1, 2, and 3 respectively; whereas their initial ratio was 1:2, 5:10.

From this it can be seen that the cultures have maintained approximately their original ratio of concentrations. A slight gain is seen in the most concentrated (dilution 3) over the more dilute

cultures (dilutions 1 and 2). Dilutions 1 and 2, however, show the same ratio at the end as at the beginning of the experiment. The conclusion to be drawn from this series is that the division rate in the more concentrated cultures is at least equal to or perhaps slightly greater than that in the more dilute cultures.

Series V.

The organisms were washed at least a dozen times over a period of several days and three times just before the experiment was started. Dilutions were made exactly as in the previous series, the

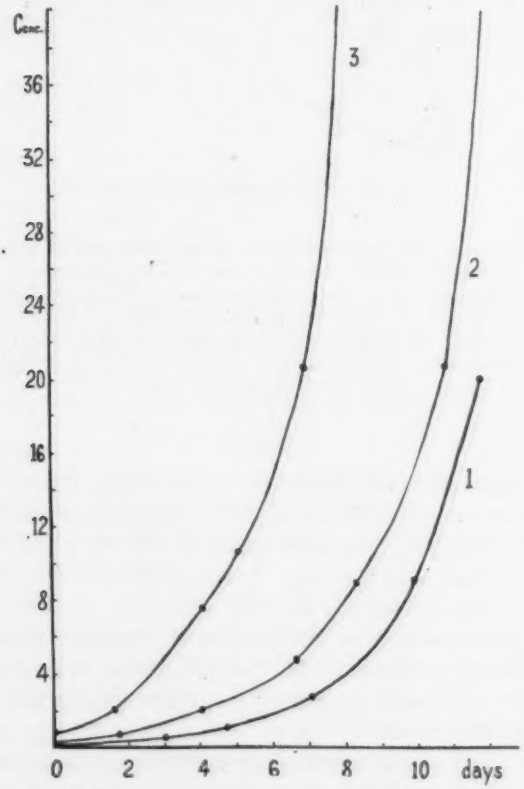

FIG. 6. Graph showing the concentrations of series V. plotted against time.

initial concentrations being .066, .165, and .66 for dilutions 1, 2, and 3 respectively. Duplicate cultures were maintained as in the previous series. The results obtained are shown in Fig. 6, and the curves are reduced to unity in Fig. 7. The increase in numbers was proportionately higher in the more dilute cultures.

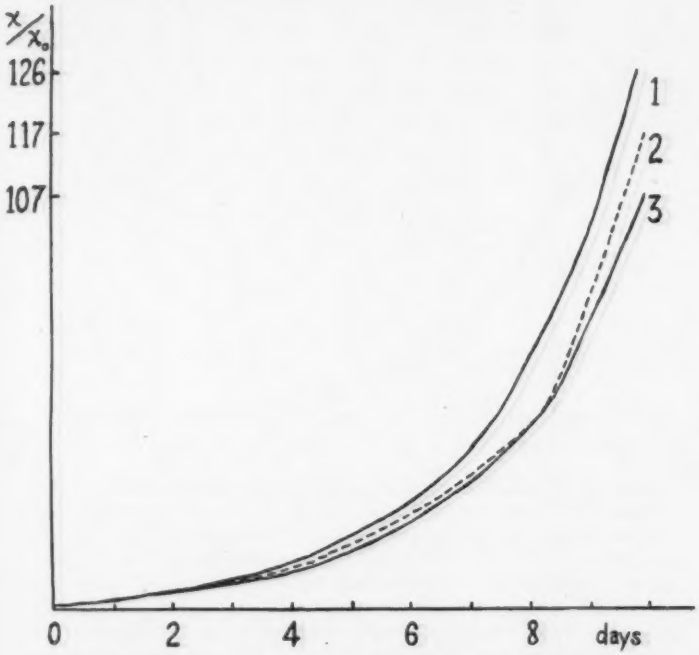

FIG. 7. Graph showing x/x_0 (x being the number present at any time, t; x_0 the initial number) of Fig. 6 (series V.) plotted against time. Note that the order of the curves is reversed from that shown in Fig. 6, denoting a greater relative increase in the most dilute culture (curve 1) at the end of the tenth day (ratio of 126:1) than in the most concentrated culture (curve 3, with a ratio of 107:1). This is directly opposed to the theory of an autocatalyst.

Series VI.

The organisms were washed, and dilutions were made as in previous series. Initial concentrations were .055, .14, and .55 in dilutions 1, 2, and 3 respectively. These were maintained in duplicate. The results are shown in Fig. 8, and the curves are reduced

to unity in Fig. 9. The rate of increase in number was proportionately the same in the lowest and in the highest dilutions for the first seven days. Then the most dilute showed a larger increase than the most concentrated. The intermediate dilution

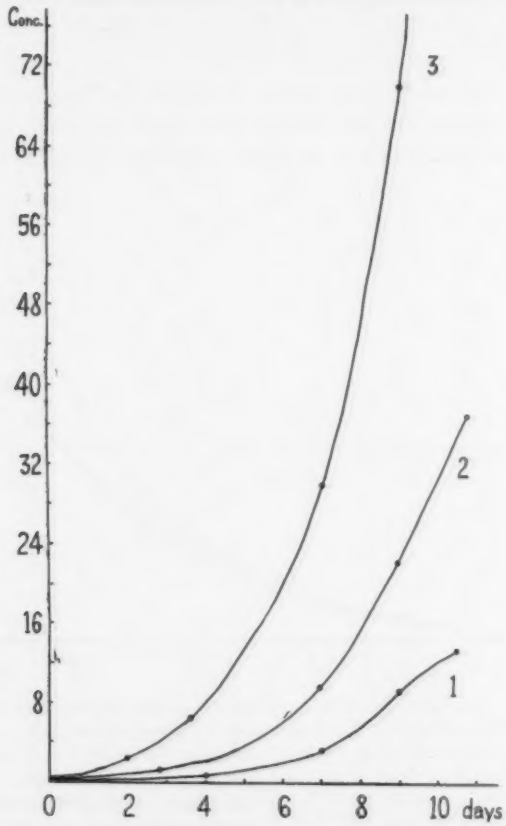

FIG. 8. Graph showing the concentrations in series VI. plotted against time.

(dilution 2) showed a higher increase on the sixth, seventh, and eighth days, a lower increase on the ninth and tenth days, and a higher increase on the eleventh day than the other dilutions. The reason for this is not known. Since no large differences were

observed in the relative amount of increase and since the minor differences were contradictory, the only conclusion to be drawn is that the concentration of organisms had no significant effect on the division rate.

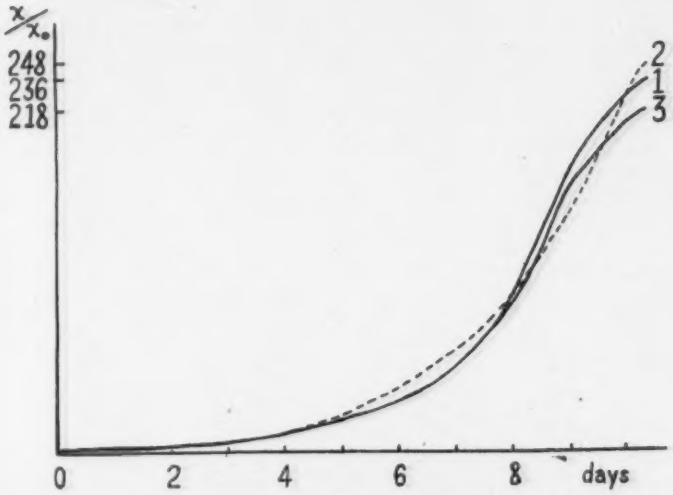

FIG. 9. Graph showing x/x_0 (x being the number present at any time t; x_0, the initial number) of the curves in Fig. 8 (series VI.) plotted against time. A difference in relative amount of increase becomes apparent between the lowest and highest dilutions after seven and one half days, the final ratios being 236:1 for the highest dilution (curve 1) and 218:1 for the lowest dilution (curve 3). The relative increase of the intermediate dilution is somewhat erratic, being higher during the sixth, seventh, and eighth days, lower during the ninth and tenth, and higher on the eleventh. These results are, of course, contradictory and indicate only that there is no large difference in division rate between cultures of low and high dilution.

DISCUSSION.

As a whole, the results of the writer's experiments with *Euglena* offer no evidence of any allelocatalytic effect. In most of the cultures, especially after the first few days, a significant difference in the opposite direction was apparent. This may be easily explained on the basis of food supply, for the more concentrated cultures would certainly use more food than the more dilute ones, leaving a lower concentration of foodstuffs in the surrounding

medium and thus directly depressing the division rate. The more concentrated cultures would also contain a higher concentration of waste products. The most probable explanation of a higher division rate in the more dilute and a lower rate in the more concentrated cultures is that the flagellates in the higher concentrations were hindered by a scant supply of food or a relatively large amount of waste products, or more likely by both these factors.

This conclusion is in accord with the experiments of Woodruff (1911, 1913) who found that waste products of one species of ciliate inhibited the division rate of the same species; and also with the observations of Greenleaf (1924, 1926) that in several ciliates a higher division rate occurred in larger volumes of medium, and that no allelocatalytic effect was present. These results are also in agreement with those of Cutler and Crump (1923*a*, *b*, 1924, 1925) for both isolation and mass cultures of *Colpidium*. Cutler and Crump (1924, 1925) explained all their results as being due to differences in food supply between large and small volumes, and their data is directly opposed to any theory of an autocatalyst.

Petersen (1929) points out that some of Cutler and Crump's (1923*a*) results indicate a statistical difference in division rate which is in favor of an allelocatalytic effect—a fact which is admitted by the authors. However, this is true only in a limited range of volume (0.5–2.5 cubic millimeters) and only when their one-animal cultures are compared to their two-, three-, and four-animal cultures. If the theory of allelocatalysis is applicable, one should expect to find differences between two- and three-, and between three- and four-animal cultures, and a very marked drop in division rate when single individuals were isolated into very large volumes. Cutler and Crump's (1923*a*, *b*) results are exactly opposite to this. If their data for two-, three-, and four-animal cultures in 2.5 to 8.5 cubic millimeters are considered, a higher division rate is obvious in the larger volumes within this range. If all volumes from .5 to 8.5 cubic millimeters are considered, however, the average division rates are about the same, regardless of whether the initial number of ciliates was one, two, three, or four. This indicates a total absence of any allelocatalytic effect. It is difficult to understand why Petersen should draw her conclu-

sions only from Cutler and Crump's small-volume cultures, when data from their other volume variations are directly contradictory.

Furthermore, in a paper which Petersen has apparently overlooked, Cutler and Crump (1924) have demonstrated a linear relationship between the average division rate and the number of initial organisms when the initial numbers are plotted in geometrical progression. In other words, they found a positive correlation between volume of medium and division rate, whereas a negative correlation between volume and division rate would be necessary to support Robertson. This relationship is shown in both isolation and mass cultures. In Cutler and Crump's earlier paper (1923*b*) this correlation was masked by the fact that the initial numbers were not plotted in geometrical progression but were given in tabular form. Even when plotted in arithmetical series they show a correlation coefficient of — .37 between division rate and initial concentration of organisms in mass cultures. This, of course, would correspond to a positive correlation of division rate and volume of medium per organism. Although this is a low correlation coefficient, it is significant because it exceeds its probable error by eight and a half times, and any correlation coefficient which exceeds its probable error may be deemed significant. In view of this overwhelming evidence it is certainly impossible to accept Petersen's (1929) interpretation of Cutler and Crump's (1923*a*, *b*, 1925) results. Their own conclusion (1924, 1925), that the division rate is higher in cultures with a lower concentration of organisms, is well founded and supported by their experiments, and is directly opposed to any theory of an autocatalyst.

Myers (1927 found that the division rate of *Paramecium* decreased with the density of population. Peterson (1929), however, shows that by selecting certain data from Myers' paper she could find some evidence for allelocatalysis. Myers, by averaging five lines, computed the mean generation time to the first division for 1, 2, 4, and 8 individuals isolated into 2, 4, 8, and 16 drops of medium. He performed three series of experiments—one series each with fresh, one-day-old, and two-day-old medium. In most cases the time to the first fission increased with the density of population. The two sets of experiments selected by Petersen are the sixteen drop cultures from the one- and two-day infusions,

although the ten other similar sets of experiments show more definite and exactly opposite results. These two sets, however, do show a difference of 2.4 hours in time to the first fission between one- and eight-animal cultures. Myers recorded the number present at the end of six, twelve and twenty-four hours in each of the five lines, thus determining the generation time of each line as six, twelve, or twenty-four hours. The mean generation time was then obtained by averaging the five lines. It is very unlikely, however, that these mean generation times are accurate because the intervals between counts (six hours) were approximately equal to the average generation time. Therefore it was impossible to obtain mean generation times that were accurate within two or three hours, especially when only five lines were considered. For example, if an organism had divided at the end of six and one half hours after isolation, the division would not have been counted until the end of the second six-hour period, and its generation time would go on record as twelve hours—an error of almost six hours or almost 100 per cent. of its true generation time of six and one half hours. In view of the small number of organisms involved, the time between observations in such isolation experiments should not be more than a fraction of the generation time of the organisms under observation, and should be much less than the expected difference which is to be measured. Since, in this case, the periods between observations were almost equal to the generation time, the value of the mean is accurate only within two or three hours, which is 33–50 per cent. of its probable true value. Therefore it is very unlikely that the 40 per cent. difference (2.4 hours) which Petersen calculated between one- and four-animal cultures has any significance at all except perhaps to show that the data which she chose from Myers' paper were entirely insufficient for such an analysis. It is also very difficult to understand how she could consider these two 16-drop series more important than the other ten sets which were performed with the same material under similar conditions, and yet gave opposite results. It is evident, therefore, that the only results of Myers (1927) which seem to indicate an allelocatalytic effect are not significant and that the interpretation placed upon these experiments by Peterson (1929) can not be valid. In view

of his results, Myers' own conclusions are logical, are supported by his evidence, and are opposed to the autocatalytic theory.

Robertson (1921*b*), in a series of experiments in which he isolated single ciliates and pairs of ciliates, chosen at random from young parent cultures, into the same volume of medium, found that, instead of twice as many organisms in the two-cell as in the single-cell cultures, there were from three to six times as many at the end of his observations. In isolations made from parent cultures over three days old, there were 2 to 2.7 as many in the two-cell cultures. Since all initial cells were isolated at random from mass cultures, the time since the last division was unknown, and the differences in the ages of the isolated cells might have been almost as much as the generation time of the organism. It can be shown also that the differences obtained by Robertson are not significant and are easily interpreted on a basis other than allelocatalysis.

Cutler and Crump (1924) found that cultures of *Colpidium* with few bacteria showed low division rates and that the organisms showed definite signs of hunger, while cultures with numerous bacteria showed a very high division rate. Other workers have tried unsuccessfully to obtain bacteria-free ciliates which would continue to reproduce. Peters (1921) believed that he was successful, but Cutler and Crump (1924) showed that his cultures were probably contaminated with a small bacillus. Parpart (1928) was successful in freeing *Paramecium* of bacteria, as were also Hargitt and Fray (1917) and Phillips (1922). Parpart makes no mention whatsoever of division in such "sterile" cultures, although he states that the organisms lived as long as five days. The only possible conclusion is that *Paramecium* does not reproduce in sterile culture medium. Hargitt and Fray (1917) and Phillips (1922) freed specimens of *Paramecium* from bacteria and then fed them known pure cultures of bacteria. Since the free-living ciliates have not been known to live without bacteria, and since there is a definite relationship between the number of bacteria and the division rate of ciliates, it would seem probable that the slow multiplication in Robertson's (1921*b*) one-cell cultures was due to lack of food in the form of bacteria.

The difference in growth rate between one- and two-animal

cultures shown in Robertson's experiments is not significant because variations obtained in his one-cell cultures are greater than the differences which are accorded to allelocatalysis. Some of his experiments, performed under supposedly identical conditions, are reanalyzed below in Table I.; since he quotes them in several publications (1921*a*, 1923, 1924*a*) they may be accepted as typical experiments. The data for the second and third columns of the table were taken from Robertson's graph.

TABLE I.

Culture Number.	Number present at First Observation.	Hours to First Observation.	Generations.	Average Generation Time to First Observation (in Hours).
238*A*	13	22	3½ approx.	6.3
240*A*	8	23	3	7.7
237*A*	8	22	3	7.3
242*A*	4	24	2	12.0

These generation times of various one-animal cultures of the *A* series show a variation of 5.7 hours, *e.g.*, culture 238*A* had an average generation time of 6.3 hours, and culture 242*A* a generation time of 12 hours. This is a variation of ± 31 per cent. from the median of 9.1 hours in cultures that, so far as known, were maintained under identical conditions.

Another of Robertson's experiments (Table II.) which he often quotes (Robertson, 1923, 1924*a*, *c*) as a proof of the allelocatalytic effect, may be compared with the data of Table I.

TABLE II.

Culture.	Initial Number.	24 hrs.	48 hrs.	Generations.	Average Gen. Time.
310*A*.....	1	2	16	4	12 hours
311*A*.....	2	3	120	6	8 hours

If the numbers of ciliates in cultures 310*A* and 311*A* are compared at the end of 48 hours, it is seen that there are 7.5 times as many cells in the two-animal culture. The average generation times are twelve hours for the one-animal culture and slightly more than eight hours for the two-animal culture. This is a dif-

ference of almost four hours, or a variation of ± 20 per cent. from the median of ten hours. Since Robertson's series of one animal cultures (Table I.), subjected to identical conditions, shows a variation of ± 31 per cent., it is impossible to consider this variation of ± 20 per cent. as being the result of any difference in division rate due to allelocatalysis. It is well within the normal variation and need not be accounted for otherwise.

Yocom (1928) isolated single individuals of *Oxytricha* into different amounts of sterile medium. He found a 14 per cent. higher division rate in the cultures of smaller volume and interpreted this as evidence of a catalyst of growth. In view of the previous discussion of bacterial food supply in this paper it is easy to see how such a small difference could arise. The food supply was less concentrated in the cultures of larger volume, and thus less readily available; hence the ciliates did not divide as rapidly as in the cultures of smaller volume.

Petersen (1929), in numerous isolation experiments with *Paramecium*, obtained results which are contradictory in that some experiments favor Robertson's view, while others are directly opposed to the theory of autocatalysis. In small volumes no acceleration was noticed in two-animal cultures, and washing produced no noticeable difference in division rate. However, when animals were isolated, washed or unwashed, singly, in two's, and in four's, into twenty drops of bacterized medium, the highest rate was observed in the four-animal cultures and the lowest in the one-animal cultures. Similar results were obtained with forty-drop cultures. Since Petersen's results argue both for and against allelocatalysis, an explanation of such contradiction must be found before her observations may be accepted as either supporting or opposing Robertson's theory.

The results of the writer's investigations offer no evidence for allelocatalysis. Except in one doubtful case, the cultures of higher concentration showed a growth rate equal to or less than that of the more dilute cultures. If an accelerating factor such as a "catalyst of growth" had been exuded during divisions, the higher concentration of this catalyst in the more concentrated cultures would have accelerated their growth, *especially after the first few divisions.* Since the growth rate was not accelerated

during the first few days it must be concluded that no such "catalyst of growth" was present.

In most cases in which growth rate of the more concentrated cultures was less than that of the more dilute the difference became more and more pronounced as the culture grew older. This indicates an inhibiting factor in the more concentrated cultures, especially after the first few days. Part of the later differences are, of course, exaggerations due to the geometrical method of increase of early differences, but the causative agent of any difference, early or late, is unknown. The fact that most of the more concentrated cultures did not grow as rapidly as the more dilute is perhaps to be explained as due to a more rapidly diminishing food supply or a more rapid accumulation of waste products in the concentrated cultures or, more likely, to both these factors. This explanation is in accord with the results of Woodruff (1913), Cutler and Crump (1923*a*, *b*), Greenleaf (1926) and others on ciliates.

At the present time Robertson's theory of allelocatalysis appears to be upheld only by some of the experiments of Petersen (1929). The theory is opposed by the investigations of Woodruff (1911, 1913), Cutler and Crump (1923*a*, *b*, 1924, 1925), Greenleaf (1924, 1926), Calkins (1926), Myers (1927), some of the results of Petersen (1929), and by the investigations of the writer. In view of this bulk of evidence against the theory, and in view of the fact that the most striking results of Robertson are shown to be insignificant, the only conclusion to be drawn is that an allelocatalytic effect is not present in protozoan cultures.

Summary.

1. A method of obtaining accurate growth curves of euglenoid mass cultures is described and its efficiency demonstrated.

2. It is shown that under the conditions described no allelocatalytic effect is present in cultures of *Euglena* sp., and it is also shown that data of other workers which have been used to support the allelocatalytic theory do not support it but indicate the absence of any allelocatalytic effect.

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HIBERNATION OF THE THIRTEEN-LINED GROUND SQUIRREL, *CITELLUS TRIDECEMLINEATUS* (MITCHILL). III. THE RISE IN RESPIRATION, HEART BEAT AND TEMPERATURE IN WAKING FROM HIBERNATION.[1]

GEORGE EDWIN JOHNSON.

Introduction.

The present study was taken up from 1915 to 1918 before the reports on waking of European rodents and bats (Horvath, '72, '78; Pembrey, '01, '03; Dubois, '96; Polimanti, '12; and others) had come to the attention of the author. While on this account many of the facts have been discovered independently by the author, the whole study has been reworked since investigation of the waking process was resumed in 1924, and it is here presented not only for its own value, but also in comparison with the work of others and as fundamental in the further study of hibernation and its possible causes. Brief reports on waking in a few individual ground squirrels have been given by Hahn ('14), Shaw ('25) and others, but the variability which has been found in the waking process has made an extended study necessary before any general description of it under different conditions of temperature and stimulation could be made.

In the first paper of this series (Johnson, '28) the conditions in hibernation and in normal activity were compared. The second paper (Johnson, '29) described two types of waking: (*a*) a relatively rapid awakening accompanied by trembling, following a *disturbing* of the animal by removing it from the nest and laying it on its side or back; (*b*) a more gradual awakening, usually without trembling, following removal *without disturbance* to a warm room, or following some handling after which it was placed back in the nest in the rolled-up position, and not removed from

[1] Contribution No. 104 from the Department of Zoölogy, Kansas State Agricultural Experiment Station, Manhattan, Kansas.

the cold room. In this type of awakening the raising of the head, the opening of the eyes and the moving forward often occurred about the same time, the state of hibernation apparently passing over gradually into normal sleep from which the animal awoke from some stimulus, external or internal. This is probably the type of waking found in nature, whereas the disturbed kind is that usually described in the literature.

This paper deals with the changes in respiration, heart beat and temperature in what must be considered the disturbed type of wakening, because the taking of the heart beat involved either the unrolling, or a more vigorous handling, of the animal.

Animals and Methods.

Some *Citellus tridecemlineatus tridecemlineatus* (Mitchill) and many *C. t. pallidus* (Allen) animals were used. The lower rate of heart beat which could not be obtained with a stetoscope, was secured by the use of a needle. At a rate of about 100 beats a minute the needle was removed and the stethoscope used. Some records were also taken by means of an electrocardiograph. It was hoped to obtain records of rise of heart beat in *undisturbed,* rolled-up animals with the electrocardiograph, but the placing of electrodes on the forelegs above the elbows apparently disturbed them even more than the needle, which could be inserted readily through the thoracic wall into the heart muscle after gently unrolling the animal and laying it on its back. For this reason the needle method appeared preferable and most of the heart beat records were taken with it. Temperatures were taken with small especially constructed thermometers but usually thermoelectrically, chiefly in the food pouch because this involved almost no stimulation. (See Johnson, '29, for further details of methods, and see discussion of Fig. 3, this paper, for further reasons for using pouch temperatures. As methods were modified in the progress of the work some discussion of them will be given within the paper.)

Miss Virginia Hanawalt, Messrs. Earl Herrick, E. Duane Sayles, Robert T. Hill and Mrs. Joanna Challans have assisted in various phases of the work. Dr. Ralph Major, of the University of Kansas Medical School, kindly extended the use of an

electrocardiograph and he and his assistants aided in taking some heart beat records with this apparatus.

CHANGES IN RESPIRATION RATE IN WAKING FROM HIBERNATION.

From a rate of one half to four a minute in deep hibernation (Cf. Johnson, '29) the respiration increased distinctly in rate when the animal was disturbed and, as is seen in the graphs in Fig. 1, this increase usually continued till near the time of opening of the eyes, when very commonly the rate was about 150 a minute and usually ranged between 100 and 200 a minute. At about the time of opening of the eyes the respiration rate often fell distinctly if the animal was quiet. The very rapid shallow inspirations common in normal excited animals were not observed in awakening from hibernation. The graphs (Fig. 1, I.) show much variation in the rate of increase from minute to minute. This irregularity suggests the presence of a coarsely adjusted respiratory regulating mechanism, stimulation of which may produce over-aëration of the blood followed by decreased respiration for several seconds or even minutes. Such periods of decreased respiration following rapid respiration sometimes lasted as long as 30 or 40 minutes in animals awaking undisturbed in a warm room. No graphs are shown of these animals because heart beat and temperature records were not taken of them.

A definite correlation between rate of increase of respiration and higher room temperature is seen in the three groups of graphs in Fig. 1. The curves in I., *a*, taken in a room of about 2 to 12° C. rise slowly; those in I., *b*, taken in a room of 15 to 22° C., rise more rapidly; and those in I., *c*, from a room of 25 to 35° C., rise most rapidly.

Simultaneously with respiration there was taken the heart beat (Fig. 1, II., *a, b, c*) and temperature (Fig. 1, III., *a, b, c*). These animals were therefore disturbed slightly by a thermocouple in the food pouch and more by being laid on the back so that the needle might be inserted into the heart. They were also exposed more to the surrounding warm air in *b* and *c* (curves *E–L*) than if they could have been left rolled up. To avoid these sources of stimulation some records of respiration and temperature alone were taken, with the animal replaced in the nest rolled up in its

natural position after the thermocouple was gently inserted into the food pouch. The head therefore was underneath and next to the cold wood shavings which constituted the nest. One of these

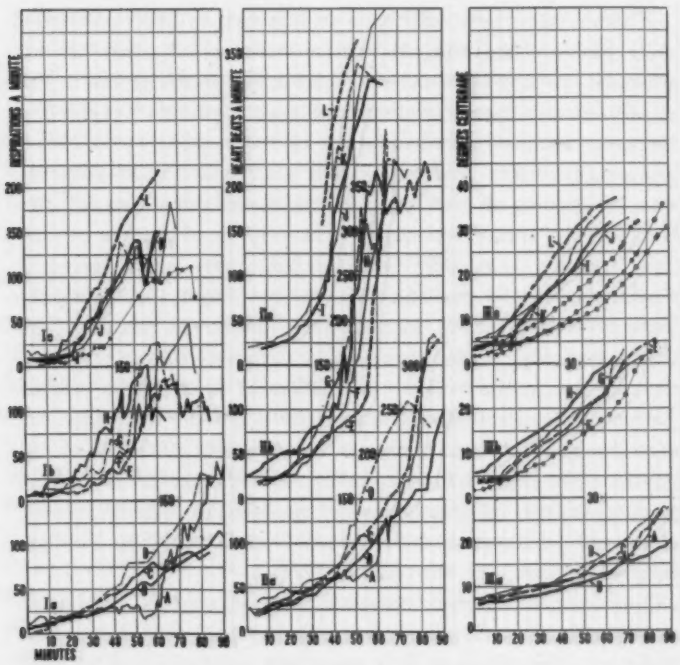

FIG. 1. Graphs of increase in respiration (I.), in heart beat (II.) and in temperature (III.), in a cold room of about 2° to 10° C. (*a*), in a room of about 15° to 22° C. (*b*), in a warm room of about 25° to 35° C (*c*). The curves belonging to one animal are designated by the same capital letter, thus I., *a A*, II., *a A* and III., *a A* represent the respiration, heart beat and temperature increases respectively in one waking in the cold room of the animal *A*. The fine lines connecting the rings in I., *c*, II., *b* and II., *c* are graphs of animals waking relatively undisturbed (thermocouple gently placed in the food pouch and the animal replaced rolled up in the nest). In the other graphs the animals were disturbed by being straightened out and by taking of heart beat in addition to temperature.

curves of relatively undisturbed awakening in a room of 23° C. was between *E* and *G* (Fig. 1, I., *b*). One in a room of 29° C. almost paralleled, but rose later than, *I* (Fig. 1, I., *c*). Another

showed almost no rise for 50 minutes, then rose parallel to *I* in Fig. 1, I., *c*. A fourth, which rose about half way between the two mentioned, is shown with dots within circles in Fig. 1, I., *c*. The rolled-up position and the probable shortage of air together with the lack of continued stimulation, because of its normal position, probably accounted for the slow rise of the curve at first. Respiration curves of animals not disturbed but merely brought to a room of warm temperature lagged from about five to twelve minutes behind those shown, but paralleled them when the rise once started. In some such animals there were long periods of very slight respiration lasting from 10 to 20 or more minutes.

Changes in Heart Beat in Waking.

The heart beat of animals left in the cold room after inserting a needle through the thoracic wall into the heart sometimes failed to rise or even fell rarely in rate for a short time, but usually there was a gradual increase at first followed by a much greater increase in the latter part of the waking process as in Fig. 1, II., *a*. A few animals had a rapid rise within 20 minutes of the time they were disturbed. Such an early rise usually indicated that the animal was not very torpid.

When animals were transferred to a warm room of about 20° C. (Fig. 1, II., *b*) the rapid rise in heart beat and the peak of its rise both came sooner than in the cold room. The rise was still more rapid and the peak reached still earlier in animals taken to a room of about 29° C. (Fig. 1, II., *c*). Under all conditions of waking the heart beat was very rapid about or a little after the time of opening of the eyes and some records of 400 beats a minute were taken with a stethoscope. The rate fell shortly after this. The very rapid increase in rate just before opening of the eyes was not produced by holding the animal because it appeared in those animals which were not held before the eyes were opened. It did not appear to be produced by the pressure of the stethoscope against the thorax, because the same rise occurred in the records taken with the electrocardiograph (Fig. 5) where there was no stimulation of the animal except the pressure of the electrodes on the forelegs. Other evidence presented later in this paper also indicates that stimulation is effective only to a limited extent in

speeding up the waking process and it appears very probably that there is always quite a marked rise in heart beat at this time in the disturbed type of awaking even if there is no stimulation at and just before the opening of the eyes.

In Fig. 1, II., *c*, *K* and *L*, two records taken with the stethoscope only are given, showing that the heart beat taken with a needle is similar in the latter part of the waking process at least to that in animals on which no needle was used.

THE RISE OF TEMPERATURE IN WAKING FROM HIBERNATION.

a. Comparisons of Œsophageal, Food Pouch, Deep Rectal and Anal Temperatures.—Graphs made of temperatures taken simultaneously in different regions of the body during waking from hibernation confirmed the observations of Dubois ('96) and others that the region near the heart warms up most rapidly. This is evident in Fig. 2, where it is seen that the deep œsophageal temperature (thermocouple inserted 5 cm. in from front of mouth, which would be to a point near the heart) rose slightly more rapidly than the temperature taken in the lower food pouch, as the animal lay on its side, which follows it very closely. The deep rectal temperature taken at a depth of 4 cm. and the anal temperature taken at a depth of 1.0 to 1.5 cm. showed almost no increase for some minutes and both lagged far behind the œsophageal and pouch temperatures until these two had almost reached their maximum some time after the animal opened its eyes and was able to move about. The anal temperature rose more slowly than the deep rectal. At other depths both in the œsophagus and in the rectum or colon slightly different curves than those shown would result. What is strikingly shown by this and five other graphs that agree with it is that the source of heat is in the thorax and as the heart and respiratory muscles are the ones which are contracting, it is evident that they are producing the heat. It is also clear that there is a marked distribution of this heat in the anterior part of the body since the pouch temperature follows that of the deep œsophagus closely but that there is a very slow diffusion of the heat to the abdomen, especially to the posterior abdomen, probably through a lack of circulation there. It is therefore a question where the temperature should be taken during the waking process.

Five other sets of graphs agree with those shown in Fig. 2 in that the pouch temperature is close to that of the œsophagus (maximum differences of 2°, 2°, 3°, 3° and 3°, respectively) in all cases

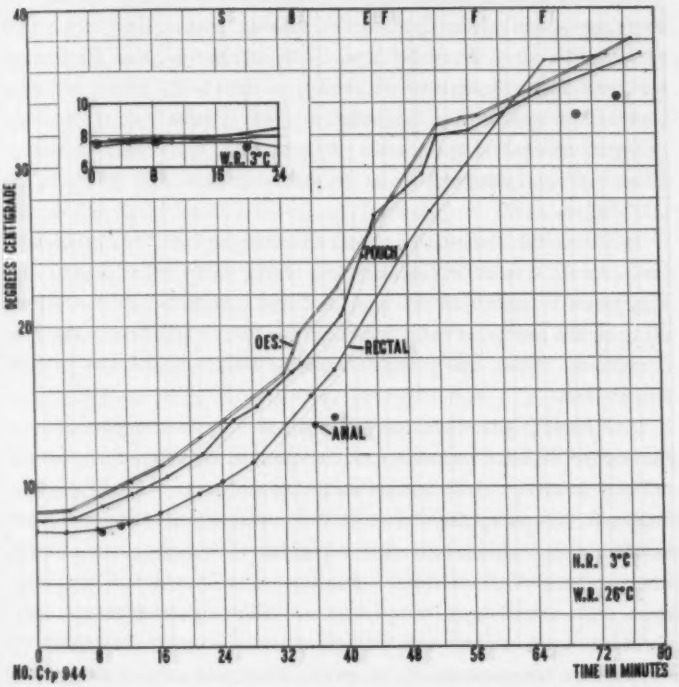

FIG. 2. Curves of the rise in temperature in the œsophagus (*œs.*) at a point 60 mm. in from the tip of the nose, in the food pouch (pouch), 43 mm. in the rectum (rectal), and 10–12 mm. in the anus (anal), all in one waking of *C. t. pallidus* 944. The insert gives observations in the cold room in which the animal was hibernating; and the main graph, the observations in the warm room. *H. R.*, hibernating room; *W. R.*, waking room; *S*, struggling; *B*, deep rapid breathing; *E*, eyes opened; *F*, fighting so that it had to be held or held down against the nest for the observation at that time. Note that the temperature of the pouch follows that of the œsophagus more closely than do the other two.

following the œsophageal temperature more closely than did deep rectal or anal temperatures. In Fig. 3 the anal temperature is almost the same as the deep rectal in the cold room (see insert,

Fig. 2) and also for a time after removal to a warm room (main graph, Fig. 2). Only one of the other five graphs agrees with this. Four of them show that the anal temperature rose somewhat more rapidly at first (probably affected by the warmth of the room) and rather late in the waking period the deep rectal temperature rose above the anal. On account of this fluctuation and even more on account of the lag in the curves which prevents them from registering a change in heat production in waking, temperatures taken in the anus or rectum are not nearly so indicative of internal temperature as are either those of the pouch or the œsophagus.

In Fig. 2 the rise of the pouch and rectal above the œsophageal temperature was an irregularity not found in the other graphs and was probably produced by some unusual conditions, as the struggling of the animal at these times, or the use of a different thermocouple, one which was protected against biting, in the last part of this record.

It is evident that the deep œsophageal region near the heart is the center of heat formation and temperatures taken there would be very valuable if the animal were not disturbed in taking them. However, when records of relatively undisturbed awakening are desired œsophageal temperatures are out of the question. Pouch temperatures, while affected somewhat more by external temperatures than œsophageal temperatures, following œsophageal temperatures quite closely and doubtless are even more indicative of the internal temperature of the animal than is shown in the graphs which include also the œsophageal temperature, because pouch temperatures when taken alone can be secured with practically no handling and also because the thermocouple in the œsophagus stimulates the heart and respiratory muscles thereby causing the œsophageal temperature to rise abnormally fast, especially early in the waking period when waking is otherwise usually slow. It seems therefore that pouch temperatures taken with care give the rise of temperature in waking (produced by warming the animal or by disturbing it slightly at first) more accurately than does any other.

b. Rise of Temperature in Waking in a Cold Room of About 2° to 10° C.—Slight handling and the insertion of a thermometer

bulb in the cheek pouch would produce waking in the cold room, and even very gently inserting a fine thermocouple into the cheek pouch would often but not always produce waking. In these studies the animal was held only when necessary and only late in waking. The process of waking in a cold room was studied in 12 graphs, four of which are given in Fig. 1, III., *a*. These four are selected to show the various types of curves rather than the most prevailing type. There was usually a slow rise in temperature for some time, then a rapid rise towards normal temperature, which was then approached more gradually, giving an S-curve somewhat similar to those illustrated by Dubois ('96) for the marmot. The temperature finally became fairly stationary if the animal was undisturbed, or continued to rise for some time if handled considerably (see Johnson, '28, for effect of handling). Variation in some of the waking curves was observed. In one the temperature rose almost uniformly during the whole period. In another there were two periods of rapid rise. In some graphs and in some records of not awakening where the animal was removed to a colder room the pouch temperature fell at first, and the internal temperature probably also did in these cases for the animals became more inert. When œsophageal temperatures were taken they rose steadily from the first because of the stimulation produced by the thermocouple, even when the pouch temperature was found to drop at first.

c. Rise of Temperature in Waking in a Room of 15° to 22° C. —Sixteen graphs were made of waking in a room of intermediate temperature, four of which are shown in Fig. 1, III., *b*. The effect of the warm surrounding temperature was evidenced by an increase in the rate of rise of the animal's temperature within a few minutes after the animal was removed from hibernating quarters to the warmer room. The rate of rise was not always rapid at first (Fig. 1, III., *F*), but in some cases it was slightly more rapid than near the time the animal had reached the temperature of the room (*H* and *E*), and quite frequently the curve approached a straight line.

To determine to what extent the rapid rise of temperature of the animal in a warm room was caused by the higher external temperature two dead animals were removed from the cold room

to a warm room of about 25° C. One showed a marked rise in pouch temperature very similar to curves *E*, *G* and *H* (Fig. 1, III.) for the first 25 minutes. In the other the anal and pouch temperatures rose very close together and more rapidly at first than the deep œsophageal and deep rectal (4 cm.), but all rose more slowly than *E* and *G*. In all cases as the room temperature was approached the rise became slower.

From these and one more record in a warmer room, it is clear that the rapid rise in temperature of a torpid animal for the first few minutes after it is brought into a warm room is produced chiefly by the higher external temperature. As time passes the metabolism is greatly increased and becomes the chief cause and finally the only cause of further warming of the animal.

From the latest records taken it appears that curve *F* is characteristic of temperatures taken in a food pouch under the animal whereas curves *E*, *G* and *H* are more typical of those taken in the food pouch which was away from the nest. The four curves (*E–H*) were taken with the animals lying on the back with the food pouch away from the nest except probably *F*. Curve *F* is also quite similar to deep œsophageal temperatures as seen in seven graphs. An eighth graph of œsophageal temperatures formed a straight line.

d. Rise of Temperature in a Room of About 25° to 35° C.—Eighteen pouch temperature graphs of animals transferred from the refrigeration room to a warm room were made and studied. Some of the different types found are illustrated in Fig. 1, III., *I*, *J*, *K*, and *L*. A few of the curves (see *I*) had a period of rapid rise at first and one near the time of opening the eyes. Later work indicated that this is produced when the pouch is rather open causing warm air to enter the pouch. When a thin thermocouple was used and precautions taken not to force the mouth open most of the curves formed almost a straight line (*L*) or sometimes they rose slowly for a few minutes and then gradually more rapidly (*J*, *K*), especially shortly before opening the eyes, until the animal was awake, when the rate of rise slowed down and finally ceased.

e. Rise of Temperature in Relatively Undisturbed Awakening.—After gently inserting a thin flexible thermocouple into the food pouch of a torpid animal it was replaced in its nest in the rolled up

position. This was done just before or just after removing the animal to a warm room. Heart beat was not taken in these animals. The temperature records are given in Fig. 1, III, *b* and *c* (dots connected by fine lines). With the head underneath against the cold nest and also with lack of stimulation from unusual position the rate of rise of temperature is quite slow at first resembling somewhat the curves of temperature of waking in a cold room.

Correlation of Respiration, Heart Beat and Temperature Rise in Waking from Hibernation.

In order to study the relations between the rise in respiration, heart beat and temperature, graphs of all three were combined from a single waking process initiated by slight handling or transference to a warm room. These combination graphs show correlations between the three curves, which rise more or less together. Waking in a cold room (0° to 8° C.) showed a very gradual rise in heart beat and respiration following insertion of a thermocouple for taking temperature and of a needle for counting heart beat (Fig. 3). The rise in temperature was even slower. In Ctp 128 the temperature fell for about the first seven minutes during which temperature was taken, for the animal had been transferred from a surrounding temperature of 6° C. to a waking room temperature of 0.2° C. (rising to 4° C. near the end of the awakening period) but the increasing heart beat and respiration maintained the temperature of the animal for the next ten minutes after which there was a slow but gradual rise similar to that in Ctp 740. In both of the graphs in Fig. 3 there are some irregularities in the temperature rise. In the curve for Ctp 740 the rapid rise at 68 and 71 minutes was evidently produced by vigorous fighting by the animal against the insertion of the thermocouple into its pouch. Ctp 128 was biting and fighting fiercely with the observer's hand just before it opened its eyes at 108 minutes, resulting in an unusual rise in pouch temperature followed by a drop such as was seen in no other graph. It is evident that when an animal is vigorously using its jaw muscles that an unusually high temperature might prevail in the cheek pouch for a few minutes, but the distribution of this heat over the body would soon cause a drop in the pouch temperature to near the average temperature of the

anterior portion of the body. In the case of Ctp 740 the animal returned into hibernation and a second waking record was obtained under almost the same conditions of temperature. The graph was almost identical to that shown in figure 3.

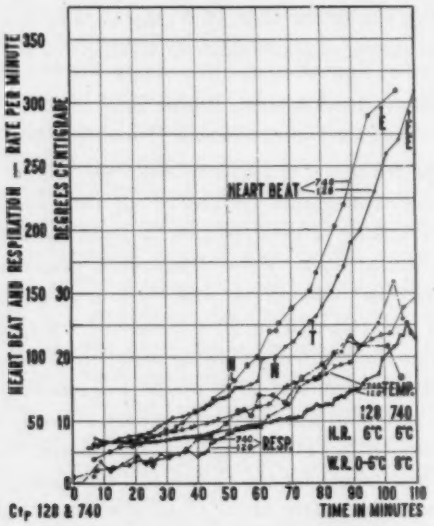

FIG. 3. Curves of rise in respiration, heart beat and temperature combined for study of correlations between them. Both *C. t. pallidus* 128 and 740 were removed from a refrigerator (H. R.) of 6° C. at 0 minutes and observations were immediately begun in rooms of 0° (rising to 6° by end of the record) and 8° C. respectively. *N*, needle removed and stethoscope used; *T*, trying to turn over; *E*, eyes opened; *F*, fighting. The dots on each graph indicate the actual readings taken.

Figure 4 gives the records of two animals waking simultaneously in a room of 30° C. under the same conditions except that no needle was used on animal Ctp 932. A general correspondence in rate of rise is seen for the three processes in each animal and also between those of the two animals. Ctp 932 was distributed five minutes later than Ctp 947 but it had a higher initial temperature so that its waking process was slightly the more rapid of the two. If the curves of Ctp 932 were moved to the right until the initial temperatures of the two animals correspond then the temperature

and heart beat of the two would be almost identical. The respiration curve of Ctp 932 would lag behind at first but be higher at the end than that of Ctp 947.

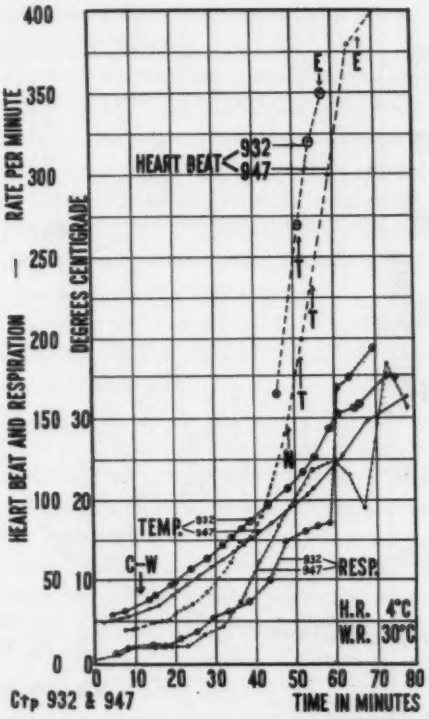

FIG. 4. Curves of rise in respiration, heart beat and temperature of *C. t. pallidus* 932 and 947 taken at the same time. The heart beat of 947 was taken with a needle to *N*, then with a stethoscope. A stethoscope alone was used on 932. *H. R.*, hibernating room; *C. W.*, point of transfer of animals from a room of 10° C. to a warm room (*W. R.*) of 30° C.; *T*, trying to turn over on feet; *E*, eyes opened.

Figure 5 illustrates two cases of waking of *C. t. pallidus* animals in which the heart beat was determined with an electrocardiograph. The electrodes were wound about the fore legs of Ctp 734 three minutes before the beginning of the record as given in the graph. The animal was transferred from a temperature of

7° to one of 17° C. at three minutes in Fig. 5. When the electrodes were placed on the fore legs the animal moved only very slightly but the respiration rose from an average of about two to

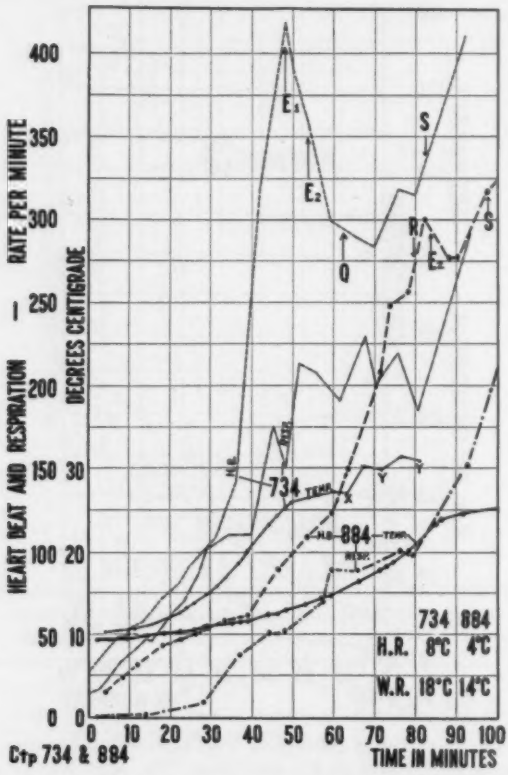

FIG. 5. Curves of rise in respiration, heart beat and temperature of *C. t. pallidus* 734 and 884, in which the heart beat was determined with the aid of an electrocardiograph. Both animals were straightened out, electrodes placed on the fore legs, thermocouple inserted into the food pouch and the animal laid on its back or side in the nest. *H. R.*, hibernation room; *W. R.*, waking room; E_1, first eye open; E_2, both eyes open; *Q*, quiet; *S*, struggling; *R*, righting, turning over on feet; *x*, thermocouple partly out of food pouch; *y*, thermocouple probably partly out.

seventeen a minute. Owing to the fact that all the observations were made by the author unaided and also to the fact that the

thermocouple did not remain completely in the pouch after the animal opened its eyes the temperature curve is not smooth as in practically all other graphs. It should be noted that this record is carried further past the opening of the eyes than in the other graphs. This accounts for the fluctuations in heart beat and respiration in the last 50 minutes.

Ctp 884 was removed from an outdoor temperature of 4° to a room temperature of 13° C. at the beginning of the record shown in Fig. 5. This animal was not disturbed much by the electrodes being placed on the fore legs. Probably partly on this account and partly on account of the colder room it woke more slowly than Ctp 734. However, internal conditions may have been influential also, as indicated in some other waking records in which an animal would wake much less readily than usual.

It is to be noted in all cases (Figs. 1, 3, 4) that the heart beat rises very rapidly once it has reached 200 or 250 a minute shortly before the opening of the eyes.

Since some observers (*e.g.*, Hall, '32) have intimated that a rapid increase in respiration as soon as the animal is disturbed is the cause of waking, an attempt was made to determine whether heart beat or respiration took the lead in waking. Fourteen graphs of waking in a cold room (1° to 12° C.) show the heart beat and respiration rising before the temperature in practically all cases. It was difficult to determine accurately whether heart beat or respiration rose first, but in about eight or nine cases the heart beat appeared to rise first, and in two or three cases respiration appeared to rise before the heart beat.

In the warm room of about 29° C. the temperature rose first in practically all of the eight graphs studied, and heart beat rose before respiration in all but one case. In a room of about 20° C. the temperature rose first in four out of eleven cases, temperature and heart beat rose together in three others and in one or two cases the heart beat rose first. Respiration was usually last to rise (seven in eleven cases). In two or three cases all three rose at about the same time.

In an electrocardiograph record of a *C. t. pallidus* the heart beat appeared to rise before the respiration when disturbed outdoors (5° C.) and fell first of the two as the animal went back into

deep hibernation. Four hours later when removed to a room of 18° C. heart beat again rose in advance of respiration. In this animal the heart missed a beat more or less frequently, as seen in Fig. 6 which is shown here because the missing of beats was not uncommon and sometimes caused abnormal awakening. (No such records have been included elsewhere in this paper.) After reaching a rate of 63 a minute at 7:25 P.M. the rate fell to 50, 44 and 20 a minute at 7:29, 7:35 and 7:42 P. M., respectively (see Fig. 6, *a*, *b*, *c*).

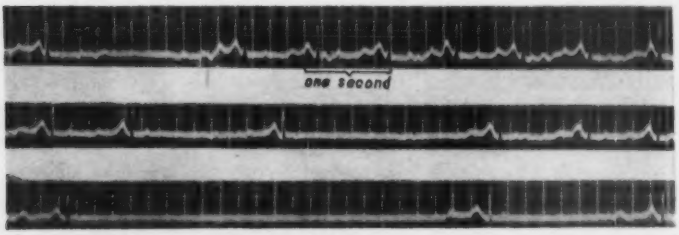

FIG. 6. Electrocardiograms of Ctp 938 showing how heart beat may fall abnormally in rate from a missing of beats after waking from hibernation has begun.

The respiration increased some after the heart beat had begun to fall, but 18 minutes after the highest heart beat the respiration had reached its highest point and took a rapid fall, remaining low in spite of the gradual warming of the animal by the room temperature (18° C.).

On a previous day (on which it showed no irregularity of heart beat) the same animal after partially awakening in a warm room showed an earlier drop in heart beat rate than in respiration when the room was rapidly cooled. During two hours of gradual warming of the room from 12° to 22° C. the heart beat rose gradually from 12 to 36 while the respiration continued at about 5 a minute. Near the end of that time the respiration showed one rise to 20 and another to 15 a minute.

While it is difficult to attribute a leading rôle to either respiration or heart beat in waking, it seems that the heart usually initiated the process to a greater extent than respiration when the animal was disturbed in a cold room. In a warm room waking

was chiefly induced by a rise in body temperature followed by an increase first in heart beat and then in respiration.

In several records respiration rose when the animal was disturbed but fell again in a few minutes, whereas the heart once started to beat faster tends more to accelerate and produce waking, as if once started to beat faster it could not decrease in rate again till waking has occurred.

The Effect of Stimulation upon the Rate of Waking.

Since it was evident that waking usually followed disturbing the animal by taking its temperature even in the cold room, it was thought that stimulation of the torpid animal would show whether heart beat or respiration was first to respond and therefore leading in the waking process. The results of a number of experiments were not very clear cut. It became evident that while stimulation of some sort usually starts the waking process, the rate of waking is not increased in proportion to the increase in amount of stimulation.

Electrical stimulation (rapid make and break shocks with a Harvard inductorium using one dry cell of 1.5 volts with the secondary coil of the inductorium set at 6 cm.) was applied to four animals a total of eleven times for a few seconds each time. Heart beat increased in four cases, decreased in three, and remained the same in three. The respiration rate increased in five cases, decreased in four cases and did not change in two cases. In one animal which did not awaken the respiration fell to 0 for a minute following six out of seven stimulations. Heart beat also was retarded in three out of the seven stimulations.

Stimulation by dropping a 5 gram weight on the animal every ten seconds from a height of five inches appeared to slightly accelerate the rate of waking, but this was only slight for the opening of the eyes occurred about the same time as in the controls in three experiments of four to eight animals each.

The continuous ringing of an electric door bell suspended near the animals had no noticeable effect on three animals as compared with their controls. Ringing for six-minute periods inhibited respiration and heart beat for a minute after the bell was started for the first two ringings. In another animal which was waking

more rapidly than usual the sound of the bell did not check heart beat and apparently accelerated respiration.

Handling the animal did not always produce waking. In a room where the temperature was falling the insertion of a thin flexible thermocouple into the cheek pouch of a torpid animal did not usually cause it to wake, though a slight temporary rise in respiration and temperature was often observed. A thermometer bulb inserted into the cheek pouch produced waking. Some animals appeared to wake more readily than others, though this could often be ascribed to a difference in body temperature at the start. One castrated male ground squirrel began to awaken in the cold room, but soon respiration, heart beat and temperature all fell and it became completely torpid again. The animal was disturbed only at first.

The Effect of a Near Freezing Temperature on Waking.

Since Horvath ('81), Dubois ('96), and Pflüger and others (according to Merzbacher, '04) found that temperatures near 0° C. caused hibernating mammals to awake and since ground squirrels appeared to me to hibernate very well below 5° C., two experiments were performed to determine whether there was a difference between species in this regard. At one time six *C. t. pallidus* were transferred from a warmer part of the refrigerator to a colder part (4° at first, falling to 2° C. in 4½ hours). All were still torpid in 85 minutes, but Nos. 1 and 2, which had a higher respiration rate (20 a minute) than the others at first, were awake, and Nos. 3 and 4, which had been deeply torpid, were waking, at 4½ hours. Left over night Nos. 1 and 2 were partly torpid again at a refrigerator temperature of 1° C., and Nos. 3 and 4 were now very deeply torpid, as was also No. 5, but No. 6 was partly torpid and woke before the next day. On the third day Nos. 1, 4 and 5 were very deeply torpid at a refrigerator temperature of 0.85° C. and No. 3 was partly torpid. On the fourth day Nos. 1, 3 and 4 were torpid at a refrigerator temperature of 0.3° C.

At another time the cages of three *C. t. pallidus* were placed on the floor of the very well insulated room at an air temperature of 1° C. and three others left in the refrigerator at 2° to 3° C. All

were deeply torpid at first except that one of those removed to the floor was breathing deeper than the others. This and one other on the floor and one in the refrigerator woke within eight hours. The third one on the floor was still torpid the next morning at a room temperature of 0° C., but began to awake in the afternoon. Two in the refrigerator did not wake the first two days. Transferred to the floor one woke the following day.

While many of the animals in these experiments woke from hibernation when transferred to a room of within a degree of freezing, this did not occur to the extent claimed for the marmot by Dubois ('96), nor did this temperature hinder a return to the hibernating state in ground squirrels. An examination of his plates 4 and 5 show that the buccal temperature rose from the first. This fact suggests strongly that waking was produced partly by the mechanical stimulation involved in taking the rectal temperature and in transferring the animal to the colder room. The same two sources of stimulation were present in Horvath's ('81) experiments. Further evidence that waking from hibernation does not always occur was found in the death of a number of hibernating ground squirrels in the early part of the work when attendants permitted the room temperature to fall to or below freezing.

Discussion.

A continuance of hibernation appears to be dependent upon the absence of external and internal stimuli, for waking is readily induced by mechanical or electrical stimuli or by warming. Since some animals wake more readily than others internal conditions appear also to influence waking. This is also indicated by the awakening at intervals of a few days of many hibernating animals.

Heart beat appears to increase somewhat more than respiration at the beginning of waking and, therefore, seems to have more influence on waking than does respiration. The interesting condition of inhibition of respiration producing inhibition of heart beat in the duck shown by Dooley and Koppanyi ('27) was kept in mind during this study of waking from hibernation, but heart beat seemed to rise independently of respiration, not only in ordinary waking but also in a case where the thorax was cut open so that the animal could not secure air. This animal had a higher

rate of heart beat when it was warmed, rising from 4 to 28 a minute in 37 minutes in one animal transferred from a surrounding temperature of 4° to one of 18° C. Such an animal could probably not become much warmer than the room because of the lack of oxygen in its blood.

At no time in waking were heart beat and respiration at the same rate, but sometimes the two would occur together, a condition claimed to be common in deep torpor by Dubois ('96) in the marmot. He also thought that the heart beat was augmented when it occurred at the same time as an inspiration suggesting a causative relation of respiration to heart beat. No such increased beat of the heart was apparent when it occurred at the same time as a respiration in the torpid ground squirrel.

The rise of temperature in a torpid animal in a cold room is slow at first because respiration and heart beat increase only very gradually and, therefore, there is little increase in heat produced. It is generally considered (Bayliss, '18, p. 455) that muscular contraction is the "chief if not the only, source of heat of practical importance to the animal organism."

Since contact or electrical stimulation usually causes waking of the torpid animal, the question arises why persistence of stimulation during waking does not speed up the process greatly. That it does not do this at all in proportion to the amount of stimulation, suggests the "all or nothing" principle. This principle would not seem to hold in an exact way, but waking is nearly always completed when once started, and the rate may not be increased much by added stimuli. One reason that the rate is not modified greatly by stimulation is probably the inability of muscle to contract rapidly when cold as observed by electrical stimulation of the muscles through the skin. The acceleration of the waking process by warming and the retardation by cooling the animal support this, as does also the regaining of motility in the anterior (warmer) regions of the body at an earlier time than in the posterior (colder) portion of the body in waking. At the very beginning of waking from deep hibernation there is usually no response to even severe mechanical stimuli like cutting through the body wall, indicating that such stimuli do not pass through the central nervous system, but as waking begins and while the

animal is still cold (10–15° C.) coördinated responses to electrical and mechanical stimuli appear. These responses are sluggish but fairly vigorous, and lead an observer to expect the stimulated animals to wake up much more rapidly than the controls.

Another factor in the production of a rapid rise of metabolism may be the temperature control mechanism. This appears to be only slightly active in the first part of waking and in quiet awaking does not appear to be so active as in the latter part of stimulated awakening where the shivering and trembling probably produced by the heat regulating system doubtless aid in the production of the very rapid rise in metabolism shown in most of the graphs in this paper. That the heat regulating mechanism is not so active in animals waking undisturbed in a warm or cold room may be indicated by the almost entire absence of shivering and shaking.

The production of a torpid state in cats by means of anæsthesia by Britton ('22) and the gradual recovery that resembles waking from hibernation tends to bear out the idea that the muscles of the heart and thorax are inhibited by cold and have grades of functioning according to their temperature, which also appears to be true of cold blooded animals. Britton could not produce a state of artificial hibernation but found that the cats would either die or very slowly awake from a body temperature of about 19° C. in a room of the same temperature. An experiment in which the temperature of the ground squirrel was lowered by means of ether anæsthesia to 22.8° C. was performed by the author before he knew of Britton's work. Spontaneous awakening took place. Many interesting comparisons might be worked out between the cooling of a cat or other homoiothermal animal and of a hibernating mammal. It should be noted that Horvath ('81) found that the hibernating animal had the ability to survive when the body temperature is lowered in ice water to much below 19° C. without artificial respiration, whereas non-hibernating forms (dog, rabbit) died if subjected to temperatures below 19° C. without artificial respiration (Horvath, '76). If artificial respiration was kept up the rabbit heart stopped beating at 9° C., whereas in the hibernating animal it did not stop at 4° or even 2° C. (Horvath, '81).

SUMMARY.

The increase in respiration, heart beat and temperature as shown in graphs is very gradual at first in waking in a cold room of 0° to 8° C., whereas in a warm room of near 30° C. the process is more rapid from the first. In both cases there is a very rapid rise of all near or at the time of the opening of the eyes when the respiration rate commonly ranged between 100 and 200 a minute, and the heart beat rate usually ranged between 300 and 400 a minute, and the temperature between 20° and 34° C. The anterior part of the animal wakes more rapidly than the posterior regions, and œsophageal and food pouch temperatures indicate the increase in metabolism more accurately than deep rectal or anal temperatures. There is a general correlation between the curves of rise of respiration, heart beat and temperature. In the production of waking heart beat appears to be somewhat more effective than respiration. Waking may be induced by stimulation but its rate is not proportional to the duration or intensity of the stimulation applied, except in the case of a surrounding high temperature. Transference to a room of about 0° C. appears to stimulate waking in some but not in all torpid ground squirrels. This temperature does not prevent hibernation.

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